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THE HISTOGENESIS OF PRIMARY HEPATIC CARCINOMA

BY

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(RECEIVED FOR PUBLICATION JULY 8, 1957)

The existence of hepatocellular and cholangiocellular carcinomata, arising from liver cells and bile duct epithelium respectively, has long been recognized, although the proportions of each type have varied in different series.

Gustafson (1937) reported 39 liver cell and 11 bile duct tumours amongst 62 cases of primary liver carcinoma based on 24,400 necropsies, and Wilbur, Wood, and Willett (1944) described 45 hepatomas and four cholangiomas. This predominance of liver cell tumours is also stressed by Counseller and McIndoe (1926), Loesch (1939), and Hoyne and Kernohan (1947). On the other hand, Fried (1924) described four cholangiomas and one hepatoma, while Karsner (1911) and Cunningham (1943) reported a preponderance of cholangiomas.

The existence of a combined tumour, consisting of hepatocellular and cholangiocellular elements, has been postulated comparatively recently. Milne (1909) and Beattie and Donaldson (1912) only recognized two types, while Pirie (1921) reported five tumours of doubtful or mixed origin amongst 36 cases of primary hepatic carcinoma. Gustafson (1937) described two cases of "intermediate or dual origin" in his series and this combined carcinoma has also been described by Warvi (1944).

Allen and Lisa (1949), reporting five cases of combined liver cell and bile duct tumours amongst 35 cases of primary hepatic carcinoma, described three possible combinations, namely, separate hepato- and cholangiocellular tumours in the same liver, separate tumours which intermingle, and individual masses which display both features so intimately associated that they can only be interpreted as arising from the same site. Three of their cases fell within the last group, while Campbell (1949) described four examples of mixed tumour amongst 22 cases of spontaneous hepatocellular and cholangiocellular carcinoma in ducks.

The occurrence of three apparently mixed hepato- and cholangiocellular tumours in an unselected series of eight primary hepatic carcinomata in an undergraduate teaching museum is of interest, and histological examination of this series is considered to throw light on the question of the histogenesis of hepatic carcinomata.

Necropsy Summaries

Case 1.—Female aged 85 years. Death from congestive cardiac failure, due to coronary atheroma, myocardial fibrosis, and essential hypertension.

Liver.—Weight 4,825 g. The right lobe was almost entirely replaced by a large, lobulated bile-stained tumour showing necrosis and haemorrhage. The left lobe contained multiple small tumour nodules and the remaining liver tissue was cirrhotic. Many pulmonary metastases were present.

Microscopically the tumour was a well-differentiated hepatocellular carcinoma. The tumour cells closely resembled those of normal liver and were arranged in columns and trabeculae (Fig. 1). In places they were gathered into irregular lobules, surrounded by fibrous tissue, and bile could be seen.

Case 2.—Male aged 67 years. Death from cerebral haemorrhage due to cerebral atheroma.

Liver.—Weight not known, but the organ was considerably reduced in size and was cirrhotic. The right lobe contained a single rounded tumour, 5 cm. in diameter, showing necrosis and haemorrhage.

Microscopically the tumour was a moderately well differentiated hepatocellular carcinoma, composed of small irregular polyhedral cells arranged in solid masses. Occasional tumour giant cells were present and the stroma was scanty.

Case 3.—Male aged 62 years. Death from liver failure.

Liver.—Weight not known, but the organ was greatly increased in size. The right lobe contained a circumscribed lobulated mass of pale tumour, while smaller tumour masses were present throughout the remainder of the right lobe and the whole of the left lobe. No cirrhosis was present. The right hepatic vein was blocked by tumour and there were numerous pulmonary metastases. Microscopically the tumour was a poorly differentiated hepatocellular carcinoma.

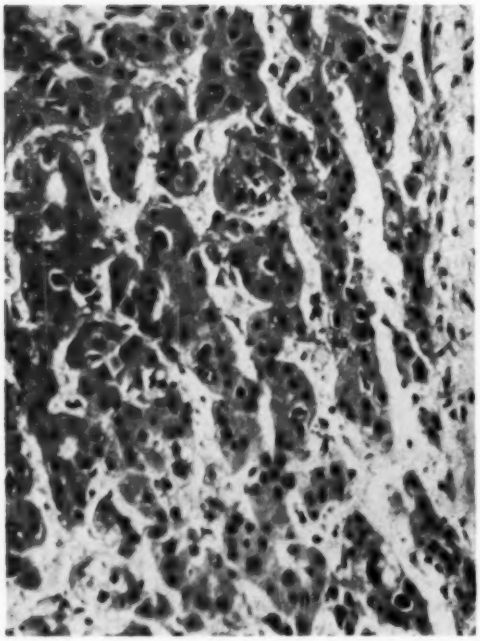

FIG. 1.—Case 1: Well-differentiated hepatocellular carcinoma showing bile. Haematoxylin and eosin × 200.

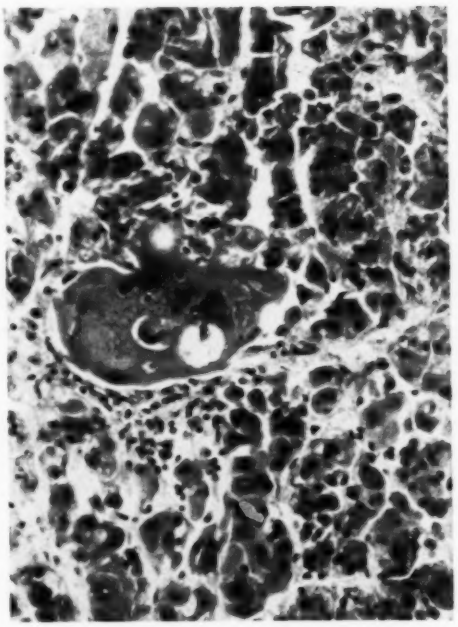

FIG. 2.—Case 3: Poorly differentiated hepatocellular carcinoma with tumour giant cells. Haematoxylin and eosin × 200.

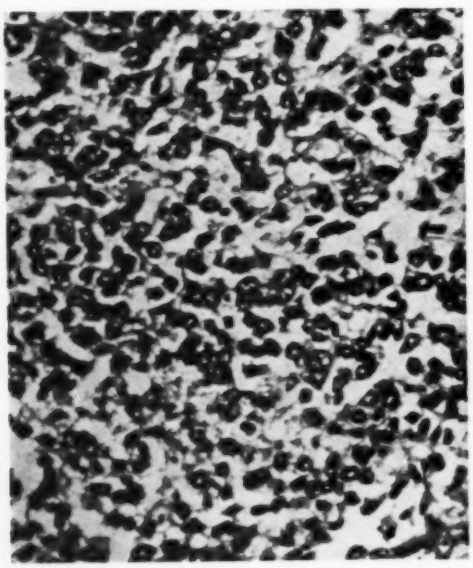

FIG. 3.—Case 4: Anaplastic carcinoma. Haematoxylin and eosin × 200.

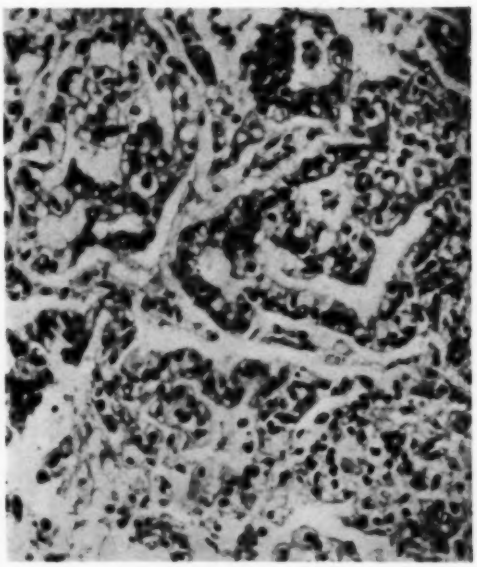

FIG. 4.—Case 4: Area with cholangiomatous appearance. Haematoxylin and eosin × 200.

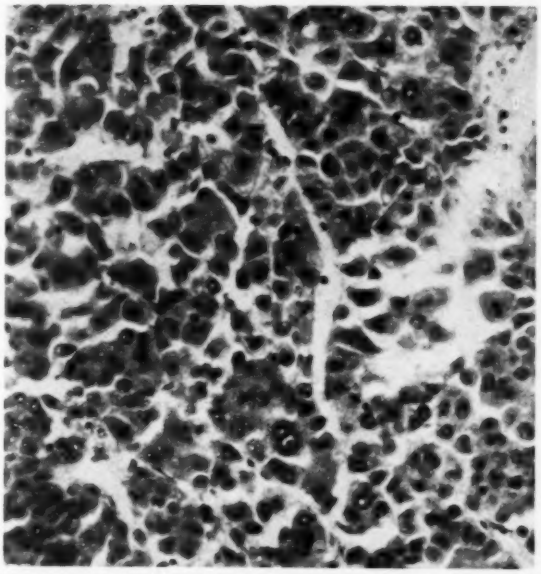

FIG. 5.—Case 5: Undifferentiated hepatocellular carcinoma. Haematoxylin and eosin × 200.

The cells tended to be arranged in columns and masses, but there was much cellular pleomorphism and there were many tumour giant cells (Fig. 2).

Case 4.—Female aged 66 years. Death from liver failure.

Liver.—Weight 4,000 g. The right lobe contained numerous closely packed pale tumour nodules, the left lobe fewer but similar masses. Both hepatic veins were distended with tumour tissue. The small amount of surviving liver was cirrhotic.

Microscopically the tumour was a predominantly anaplastic and undifferentiated carcinoma (Fig. 3), with some areas showing a cholangiomatous glandular pattern (Fig. 4).

Case 5.—Male aged 62 years. Death from liver failure due to haemochromatosis.

Liver.—Weight 3,324 g. The central part of the organ contained many nodules of pale tumour, the largest 2 cm. in diameter. Tumour in the hepatic veins extended into the inferior vena cava and the portal vein was thrombosed. The remaining liver tissue showed the typical changes of haemochromatosis.

Microscopically the tumour was predominantly an undifferentiated hepatocellular carcinoma (Fig. 5), but with areas of tumour consisting of small structures resembling bile ducts (Fig. 6).

Case 6.—No clinical details available.

Liver.—Approximately normal size and containing numerous nodules of pale tumour, the largest 1 cm. in diameter. The remaining liver tissue showed the typical changes of haemochromatosis.

Microscopically the tumour contained poorly differentiated hepatocellular carcinoma (Fig. 7) and cholangiomatous areas consisting of tubules lined by cuboidal and columnar epithelium (Fig. 8).

Case 7.—Male aged 58 years. Death from liver failure and bronchopneumonia.

Liver.—Weight 1,840 g. A mass of pale tumour, 15 cm. in diameter, was present in the lower part of the left lobe and projected from it. Smaller nodules of similar tumour surrounded the main mass, and the remainder of the liver was deeply bile stained and showed coarse irregular fibrosis. A tongue of tumour extended from the main mass into the left hepatic and common bile ducts.

Microscopically the tumour was an undifferentiated hepatocellular carcinoma with areas of differentiation into tubules suggestive of small bile ducts (Fig. 9). The surrounding liver tissue showed the changes of biliary cirrhosis.

Case 8.—Female aged 44 years. Death from suppurative cholangitis and liver failure.

Liver.—Weight unknown, but the organ was greatly enlarged and bile stained. Ramifying

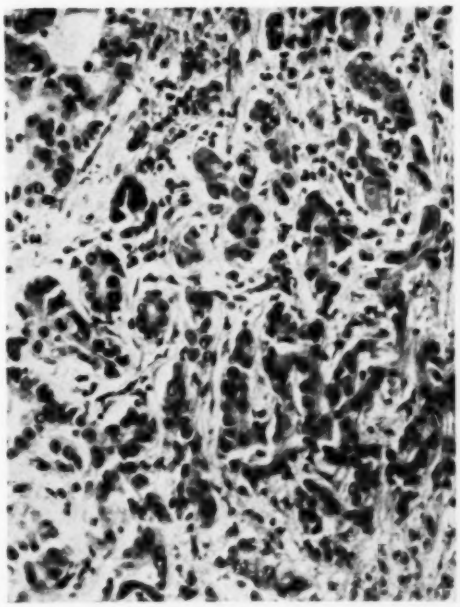

FIG. 6.—Case 5: Cholangiocellular area with structures resembling small bile ducts. Haematoxylin and eosin × 200.

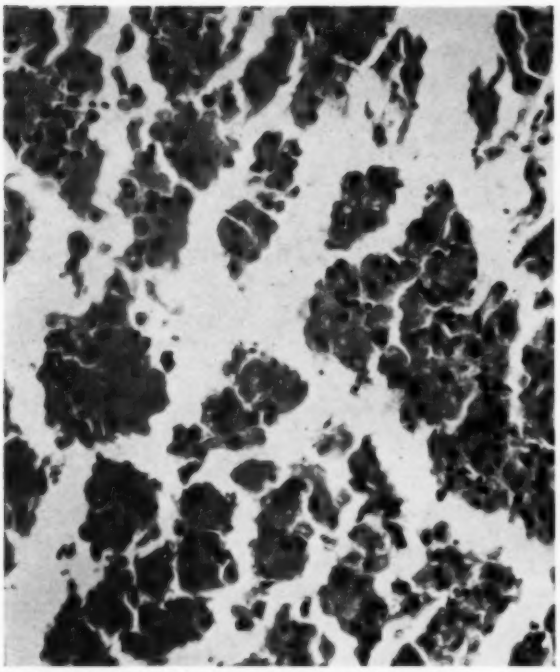

FIG. 7.—Case 6: Poorly differentiated hepatocellular carcinoma. Haematoxylin and eosin × 200.

masses of pale tumour radiated from the porta hepatis throughout the liver, and many small abscesses were present.

Microscopically the tumour was a well-differentiated, mucus-producing cholangiocellular carcinoma, consisting of large glandular spaces lined by cuboidal epithelium (Fig. 10).

The details of the cases are summarized in the following table:

Case	Sex	Age	Cirrhosis	Tumour
1	Female	85	Present	Hepatocellular
2	Male	67	,,	,,
3	,,	62	Absent	,,
4	Female	66	Present	Cholangioma
5	Male	62	Haemochromatosis	Mixed
6	—	—	,,	,,
7	Male	58	Present	,,
8	Female	44	Absent	Cholangioma

Discussion

Diverse views have been held on the histogenesis of primary hepatic carcinomata.

Milne (1909) believed that hepatic carcinomata arose from either liver cells or bile duct epithelium and that, even though liver cells and bile ducts were related embryologically, the two types of tumour were quite distinct. Yamagiwa (1911) and Beattie and Donaldson (1912) also recognized only two varieties, arising from liver parenchyma and bile duct epithelium respectively.

The existence of apparently mixed tumours, originating from both liver cells and bile ducts, was postulated by Warvi (1944) and Allen and Lisa (1949). Kinosita (1937) induced hepatic carcinomata experimentally in rats by the administration of butter yellow. He described 18 hepatomas and 16 mixed hepatomas and cholangiomas. No pure cholangiomas were produced, cholangiomatous tissue being found only with hepatomas. He therefore postulated that both liver cells and bile duct epithelium could undergo simultaneous neoplasia, although he decided later (Kinosita, 1940) that all primary liver carcinomata originated from liver parenchyma.

A case described by Koster and Kasman (1932), in which the cirrhotic liver of a 60-year-old male contained multiple apparently discrete and separate hepatomas and cholangiomas, suggests that liver parenchyma and bile duct epithelium may undergo simultaneous tumour formation.

Ewing (1940) believed that pure hepatomas and cholangiomas arose from liver cells and bile duct epithelium respectively, and that the mixed tumours either had a dual origin or had arisen from liver cells which had earlier assumed the characteristics of bile ducts. MacCallum (1904) stated that regenerating liver cells may assume, possibly temporarily, the appearance of bile ducts, and Muir (1908) described the formation of tubular structures from liver cells in a healing laceration of the liver. The latter quotes Hess (1890) as reporting that the cells of similar structures contained glycogen though less than the surrounding liver parenchyma. Muir (1908) also considered that, although true bile duct proliferation took place in cirrhosis of the liver, structures resembling bile ducts were also formed from liver cells. Mallory (1911), in a study of pre-cirrhotic liver lesions, also described the formation of tubular structures or pseudo bile ducts from liver cells, and his description of these suggests that their cells were smaller and darker than those described by MacCallum (1904) and by Muir (1908) in his healing liver laceration.

Opie (1944) produced cirrhosis and primary liver carcinomata experimentally in animals and

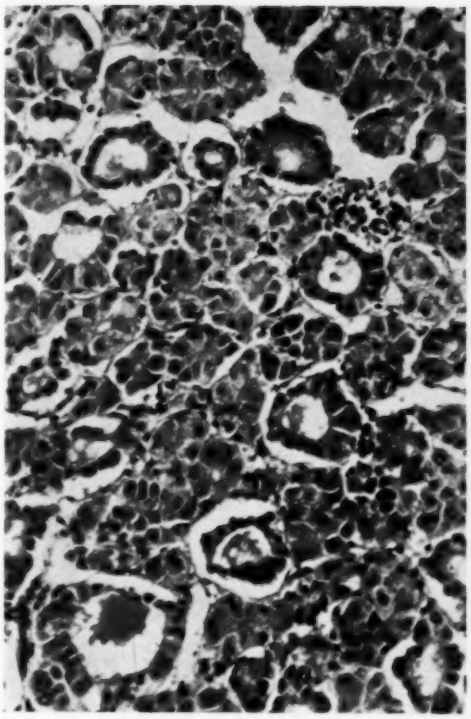

FIG. 8.—Case 6: Cholangiocellular area. Haematoxylin and eosin × 200.

described the formation of pseudo bile ducts around both biliary tracts and central veins, in which latter site they could only have arisen from liver cells. His description leaves no doubt that these new or pseudo bile ducts were everywhere derived from liver cells.

A careful search around nodules of hyperplastic liver cells in cirrhotic livers revealed the formation of pseudo bile ducts, of varying size, from regenerating liver cells (Fig. 11), and glycogen could be demonstrated in their cells, albeit with difficulty.

There is, therefore, abundant evidence that liver cells can give rise to tubular structures of varying appearance, many of which resemble bile ducts, and Mansens (1954) considers that this is due to dedifferentiation of liver cells in the presence of growing mesenchyme.

The first three cases reported here are hepatomas of progressing anaplasia and undifferentiation. The last case (Case 8) is a classical, well-differentiated cholangioma, while Case 4 is a cholangioma with large anaplastic and undifferentiated areas. Three examples of apparently mixed

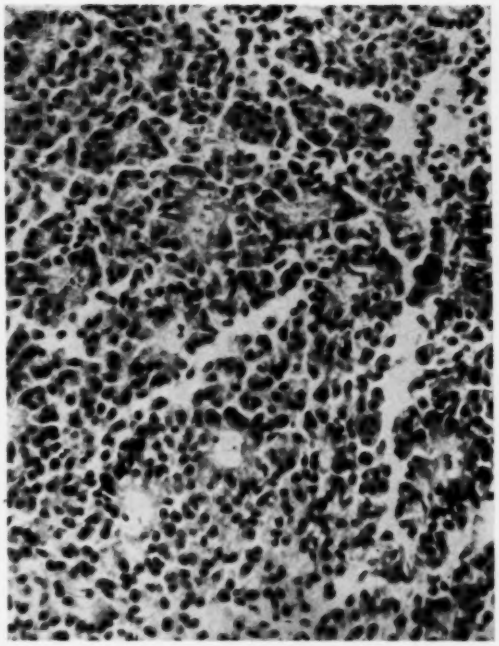

FIG. 9.—Case 7: Area showing tubular structures resembling small bile ducts. Haematoxylin and eosin × 200.

FIG. 10.—Case 8. Well-differentiated cholangiocellular carcinoma. Haematoxylin and eosin × 200.

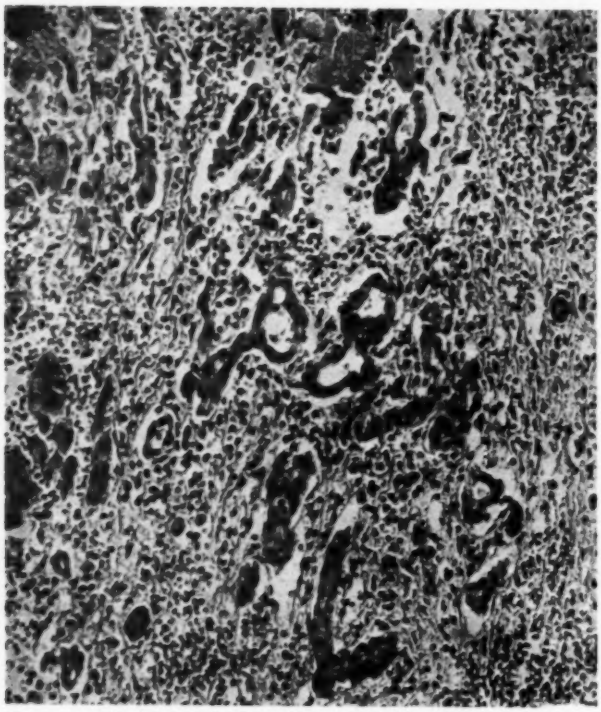

FIG. 11.—Pseudo bile ducts being formed from liver cells in a cirrhotic liver. Haematoxylin and eosin × 200.

tumours (Cases 5, 6, and 7) consist of hepatocellular tissue compounded with cholangioma-like areas containing acini, which resemble small bile ducts.

The cholangioma-like elements in Case 6 (Fig. 8) closely resemble the structures described by Muir (1908) in a healing liver laceration, and by MacCallum (1904). The same elements of Case 5 (Fig. 6) are strikingly similar to the pseudo bile ducts described by Mallory (1911) and by Opie (1944) and those illustrated in Fig. 11.

Glandular structures of varying size resembling bile ducts have been described in tumours which were considered to be pure hepatomas (Loesch, 1939; Evans, 1956). Warvi (1944), although adhering to the concept of mixed tumours, admitted that hepatomas may contain areas of pseudo-biliary structure giving a false impression of hepatocholangioma, while Hou (1956) described hepatoma-like areas in hepatic carcinomata which, he believed, had arisen almost certainly from bile ducts.

Although simultaneous neoplasia of liver cells and bile duct epithelium may occur, the conclusion must be reached that only two varieties of primary hepatic carcinoma exist, namely hepatomas and cholangiomas. The apparently mixed type are usually hepatomas with pseudo-biliary differentiation, but may be cholangiomas containing anaplastic areas morphologically resembling hepatomas.

Summary

Eight cases of primary hepatic carcinoma, three hepatocellular, two cholangiocellular, and three of apparently mixed variety, are described.

An examination of these and previously reported cases, as well as experimental data, indicate that both hepatocellular and cholangiocellular carcinoma may mimic each other and that mixed tumours, arising from both liver cells and bile duct epithelium, must be extremely rare, if they occur at all.

We wish to thank Mr. E. A. Wallington, F.I.M.L.T., and the technical staff of the Department of Morbid Anatomy for their assistance, and Mrs. P. Thomas for the photomicrographs.

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PRIMARY LIVER CELL CARCINOMA 24 YEARS AFTER INTRAVENOUS INJECTION OF THOROTRAST

BY

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Thorium dioxide, when injected intravenously, is taken up by the reticulo-endothelial system and retained indefinitely, rendering the liver and spleen radio-opaque (Radt, 1930). A 25% colloidal solution of thorium dioxide was marketed under the name of "thorotrast" and used not only for hepatolienography but for retrograde pyelography, bronchography, and angiography generally, not to mention other uses, e.g., cerebral ventriculography, arthrography, mammography, dacrocystography, antral visualization.

From the outset its tumour-forming potentialities were recognized (Stewart, Einhorn, and Illick, 1932), and the American Medical Association (1932) recommended that intravenous administration be discontinued, but for some years "thorotrast" was used by those who held that its radioactive properties were negligible. The conflicting claims were reviewed by Rigler, Koucky, and Abraham (1935) and by Orr, Popoff, Rosedale, and Stephenson (1938).

Injected into animals, "thorotrast" induces sarcomata after a latent period (Roussy, Oberling, and Guerin, 1934; Selbie, 1936). By means of the Geiger counter Taft (1937a) was able to show that the standard dosage for human hepatolienography (75 ml.) gives a gamma radiation equivalent to 1.37 micrograms of radium.* Reeves and Stuck (1938) observed that retention of "thorotrast" in the reticulo-endothelial system affords a continuous opportunity for the damaging effects of alpha radiation. These rays have a higher relative biological efficiency than beta or gamma rays, and their activity is extremely localized. The emission of alpha particles in rabbits injected with "thorotrast" was studied under the spinthariscope by Orr *et al.* (1938).

*Radium and its first two daughter isotopes emit α particles like thorium. The proportion of γ-emitting daughter isotopes in the decay products of radium and thorium to the primary isotope is sufficiently close to justify this type of comparison. The International Commission on Radiological Protection, 1954, advises that the maximum safe total body burden of radium is 0.1 microgram.

Some comfort was taken from the finding of Stenstrom and Vigness (1940) that although the "thorotrast" is retained in the tissues indefinitely, there must be some diminution of radioactivity, since radioactive elements can be demonstrated in the faeces, urine, and breath. And in a 10-year follow-up of 286 cases Yater and Coe (1943) found "no immediate or remote ill-effects of importance" in the survivors.

In 1947 MacMahon, Murphy, and Bates described a case of endothelial-cell sarcoma of the liver in a woman aged 58, 12 years after hepatolienography had been performed in the investigation of gummatous hepatitis. Since then other cases of malignancy following the injection of "thorotrast" have been published (Tables I and II). Not all the injections were intravascular (see Table I), but it was always possible to demonstrate

TABLE I

MALIGNANT TUMOURS DEVELOPING AT SITE OF THOROTRAST INJECTED EXTRAVASCULARLY

Author	Year	Sex	Age	Mode of Thorotrast Injection	Condition for which Thorotrast was Injected	Latent Period in Years	Type of Tumour
Zollinger	1949	M	64	Retrograde pyelography	Hydronephrosis	16	Spindle-cell sarcoma of renal pelvis
Rudolphi	1950	M	51	Dacrocystography	Dacrocystitis	35	Squamous cell carcinoma of lower eyelid
Hofer ..	1952	F	64	Antral visualization	Chronic sinusitis	10	Squamous cell carcinoma of antrum
Vögtlin and Minder	1952	M	47	Bronchography	Bronchiectasis	18	Squamous cell carcinoma of bronchus
Plenge and Krückemeyer	1954	F	54	Cerebral angiography	Suspected intracerebral disease	6	Sarcoma at site of injection
Gros *et al.*	1955	M	36	Antral visualization	Non-medical reasons	15	Squamous cell carcinoma of antrum

TABLE II

MALIGNANT TUMOURS DEVELOPING IN LIVER, SPLEEN, OR BONE MARROW AFTER INTRAVASCULAR INJECTIONS OF THOROTRAST

Author	Year	Sex	Age	Mode of Thorotrast Injection	Condition for which Thorotrast was Injected	Latent Period in Years	Type of Tumour
MacMahon *et al.*	1947	F	70	Hepato-lieno-graphy	Gumma of liver	12	Endothelial-cell sarcoma of liver
Horta	1953	F	?	Cerebral angio-graphy	Cerebro-vascular accident	3½	Endothelial-cell sarcoma of liver
Lüdin	1953	F	63	Arterio-graphy	Vascular disease of leg	14	Haemangio-endothel-ioma of liver and spleen
Matthes ..	1954	F	54	Hepato-lieno-graphy	Jaundice	21	Primary car-cinoma of liver
Heitmann	1954	M	39	Hepato-lieno-graphy	Echino-coccal cysts of liver	20	Carcinoma of common bile duct
Tesluk and Nordin	1955	M	68	Cerebral angio-graphy	Suspected cerebro-vascular disease	6	Haemangio-endothel-ioma of liver
Fruhling *et al.*	1955	M	49	Arterio-graphy	Vascular disease of leg	12	Haemangio-endothel-ioma of liver, spleen, and bone marrow
Grossiord *et al.*	1956	M	54	,,	Osteomye-litis of femur	21	Adenocarci-noma of liver
Roberts and Carlson	1956	F	45	,,	Arterio-venous fistulae of leg	17	Carcinoma of hepatic duct
Horta ..	1956	M	46	? Aorto-graphy	Unknown	22	Haemangio-endothel-ioma of liver
Present case	1958	F	48	Hepato-lieno-graphy	Upper abdomi-nal pain	24	Hepatoma of liver

a considerable quantity of "thorotrast" at the site of the subsequent tumour.

The case of Abrahamson, O'Connor, and Abrahamson (1950), who reported bilateral carcinoma of the lungs 16 years after hepatolienography, has been purposely omitted. At necropsy there was little "thorotrast" in the lungs, and the evidence that malignancy was in any way connected with the injection is very slight. A similar case was reported by Lloyd (1957). These cases are clearly different from that of Vögtlin and Minder (1952), where the tumour arose in the vicinity of large amounts of "thorotrast" left in the lung from an earlier bronchography.

In a recent review Horta (1956) rejected the case of Heitmann (1954) on the grounds that the bile-duct carcinoma did not arise near the "thorotrast" deposits in the liver. Roberts and Carlson (1956), reporting a similar case, stressed the concentration of "thorotrast" in the portal lymph nodes and their proximity to the bile ducts. We have therefore included both cases. However, we share Horta's reservations over the case of Grossiord, Roucayrol, Duperrat, Ceccaldi, and Meeus-Bith (1956), since their patient also had cirrhosis of the Laennec type, thus affording a stimulus to neoplasia other than radioactivity alone.

More exacting criteria are demanded by Guimaraes, Lamerton, and Christensen (1955), who question the cases of MacMahon *et al.* (1947), Zollinger (1949), and Rudolphi (1950), on the grounds that pre-existing inflammatory conditions (gummatous hepatitis, chronic pyelonephritis, dacrocystitis) may also have been factors in producing malignancy later, and by this token one would have to demur in accepting the cases of Hofer (1952) and Vögtlin and Minder (1952) as well, since each gave a history of antecedent inflammation at the site of the neoplasm.

The tables also omit, in view of inadequate data, three unpublished cases mentioned by Thomas, Henry, and Kaplan (1951) and four others referred to by Looney and Colodzin (1956).

The case recorded below seems to fulfil all the requirements of post-irradiation malignancy—a primary liver-cell carcinoma occurring in the absence of cirrhosis or pre-existing inflammation, 24 years after the intravenous administration of "thorotrast."

Case History

The patient, a woman aged 48, was admitted to hospital on April 5, 1956, because of abdominal pain, first noticed in November, 1955, when it appeared at the beginning of a normal menstrual period. At this time it was situated in the epigastrium and right loin and was described as being very severe. After a week's rest in bed, she recovered completely. Four days before admission she noticed generalized abdominal pain which again coincided with the onset of menstruation. This pain soon became localized to the epigastrium, but was also referred to the tip of the right shoulder and was aggravated by coughing and deep breathing.

In 1932, when she was 24, she was investigated at another hospital for an upper abdominal condition by the injection of some material into an arm vein (the exact dosage is unknown), followed by a number of radiographs of the abdomen. This must have been the occasion when the "thorotrast" was administered, since she had had no other injection.

On examination she looked ill and had obviously lost weight. At the time of her first admission, the

epigastrium was very tender, with marked guarding which at first prevented the palpation of an underlying mass. Later it became possible to feel a hard, irregular epigastric mass which moved on respiration.

The following investigations were carried out:

A radiograph of the abdomen showed radio-opacities in the liver, spleen, and lymph nodes, especially those of the pre-aortic group, the pattern being characteristic of hepatolienography by "thorotrast" (Fig. 1).

An oral cholecystogram was normal.

A barium meal showed a smooth pressure defect related to the whole of the lesser curve of the stomach. The appearances were similar to those produced by a large pancreatic cyst.

A blood count gave: haemoglobin 90%, W.B.C. 11,000 per c.mm. (neutrophils 88%, lymphocytes 8%, monocytes 4%).

Serum amylase was less than 100 units. Tests for occult blood were negative. Serum bilirubin (direct) was 0.1 mg.% and (indirect) 0.1 mg.%, total 0.2 mg.%. Flocculation tests gave: thymol turbidity, 0 units, thymol flocculation, 0 units, serum colloidal gold, 0 units, zinc sulphate turbidity, 1 unit.

In view of the concentration of "thorotrast" shown by the radiographs, a diagnosis of primary carcinoma of the liver was suspected. Laparotomy was undertaken, and the abdominal tumour was found to be a large liver riddled with hard white areas looking not unlike secondary deposits; careful examination of the abdominal contents failed to reveal a primary growth. A biopsy taken from the liver showed a carcinoma with a trabecular structure suggesting hepatic origin. The adjacent liver tissue contained "thorotrast" granules (Fig. 2).

The patient made a good recovery from her operation and was discharged from hospital on the fourteenth day after operation. Thereafter, her condition gradually deteriorated and she died at home on August 30, 1956.

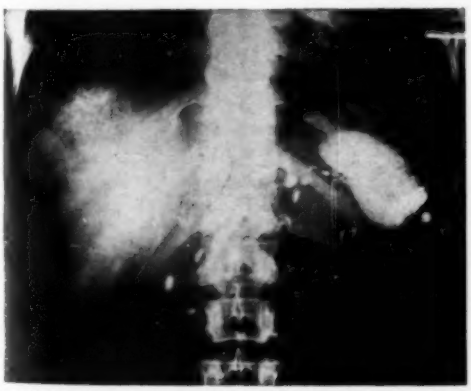

FIG. 1.—Radiograph of upper abdomen showing radio-opacity of liver, spleen (with splenunculus), portal and pancreatico-splenic lymph nodes.

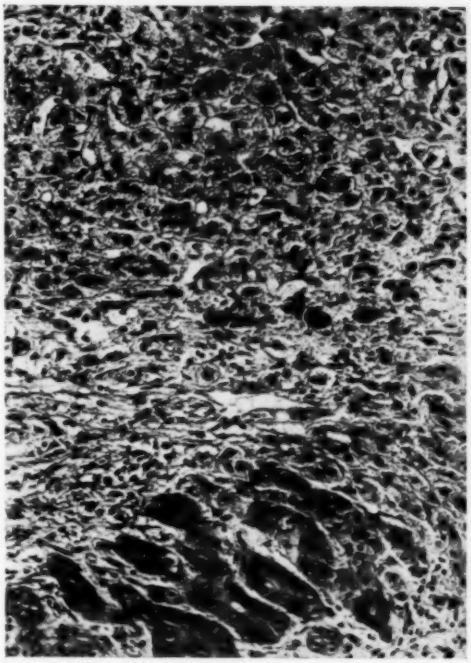

FIG. 2.—Biopsy of liver showing margin of carcinomatous nodule and "thorotrast" deposits (centre). Haematoxylin-eosin × 160.

Necropsy Report

The subject was emaciated and dehydrated. The brain, buccal cavity, upper respiratory passages, and thyroid gland were normal. The immediate cause of death was bilateral bronchopneumonia, and both lungs were riddled with metastatic carcinomatous deposits 1–2 mm. in diameter. A few of the mediastinal lymph nodes were invaded by growth. The heart showed brown atrophy; the aorta and main branches were healthy. There was mild varicosity of the lower oesophageal veins.

The peritoneal sac contained several pints of serofibrinous fluid. The liver was enlarged (79 oz.) and riddled with malignant deposits. It was not possible to determine the precise site of origin, but the right lobe was largely destroyed by confluent tumour masses, and was, if anything, reduced in size; while the left lobe appeared to be enlarged, and contained discrete deposits up to 2 cm. in diameter (Fig. 3). There was no evidence of underlying cirrhosis.

The gall bladder and bile ducts were healthy, but in the portal fissure lay a group of yellowish discrete nodules, bony hard in consistency, resembling calcified hepatic lymph nodes. Similar nodules ¼–1 cm. in diameter lay along the anterior aspect of the pancreas

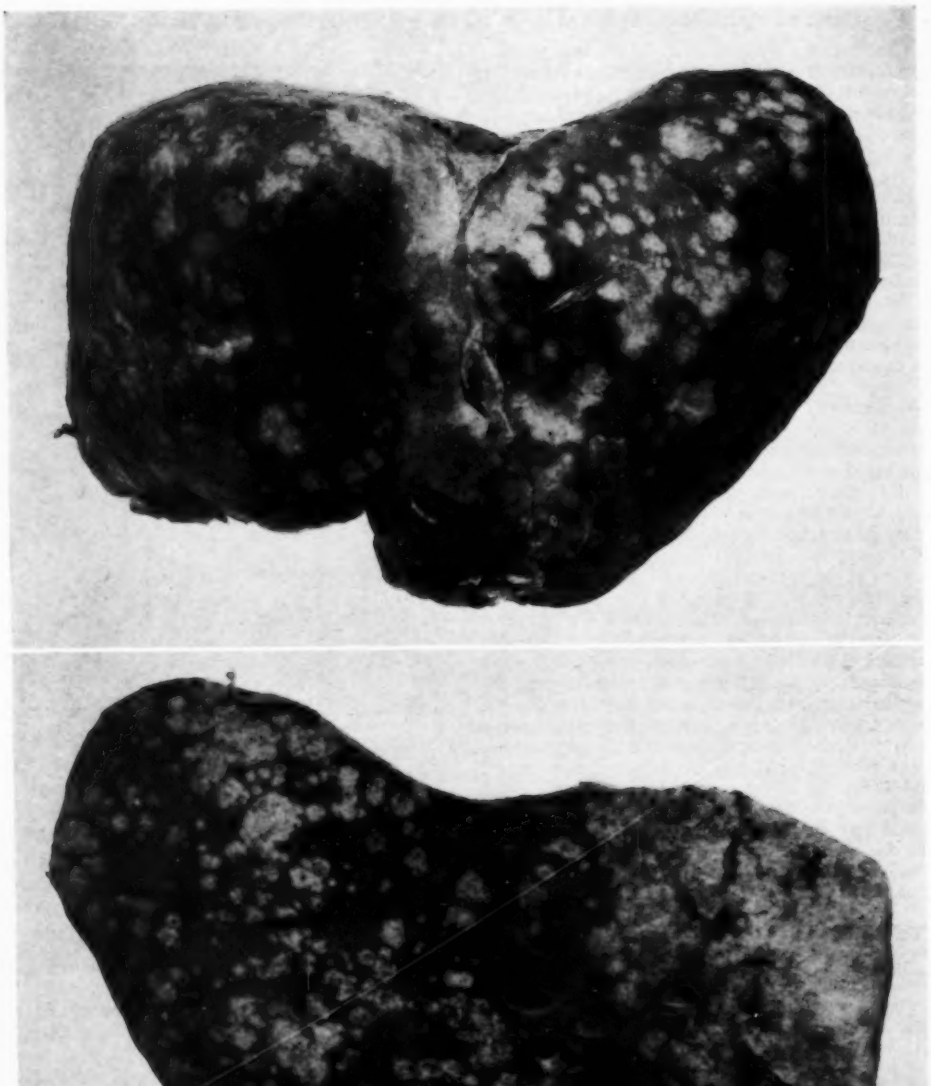

FIG. 3.—Anterior and cut surfaces of liver showing a confluent growth in the right lobe, discrete nodules in the left lobe.

and were taken to be the pancreatico-splenic lymph nodes.

The spleen was hard and shrunken (1 oz.), with a thick white capsule. The cut surface presented a remarkable appearance, the malpighian bodies being greatly enlarged and bright yellow, their colour contrasting sharply with the dark red of the pulp (Fig. 4). The splenic artery was normal.

There was a small carcinomatous deposit in each kidney—the only extrahepatic metastases in the abdomen. The pancreas, adrenals, and pelvic organs were normal, and the only other finding of note was congenital shortening of the jejunum and ileum to about one half of the usual length.

Histology

Liver.—The tumour was similar to that reported following biopsy, i.e., a primary carcinoma of the "hepatoma" type, the tumour cells occurring in short columns, with occasional attempts at acinar formation. The surviving liver parenchyma had a normal lobular pattern, and there was no real evidence of cirrhosis, although the portal canals and central veins showed a definite excess of fibrous tissue, anatomically related to the distribution of "thorotrast" (Figs. 5, 6). This was in the form of a grey, isotropic, granular material, partly extracellular but mostly contained in macrophages and conspicuously absent in the tumour tissue (Fig. 7). Liver cell degeneration, where present, was not anatomically related to the "thorotrast."

Spleen.—The yellow colour of the malpighian bodies proved to be due to massive deposits of "thorotrast" (Figs. 8, 9). Under higher magnification this was observed as an aggregation of small rounded granular clumps, each of which owed its outline to the limiting membrane of a macrophage cell, although it was rarely possible to demonstrate the nucleus. The sharp circumscription of the deposits was exaggerated by an almost total depletion of malpighian lymphoid tissue, and a general atrophy of the red pulp.

Lymph Nodes.—The hepatic and pancreatico-splenic groups required prolonged treatment with a decalcifying agent before they could be sectioned. The lymphoid tissue was completely replaced by dense fibrous tissue incorporating large quantities of extracellular "thorotrast," except at the hilum, where the transfer of granules by macrophage cells appeared to be still active (Fig. 10). There were no metastases in these nodes.

Bone Marrow.—A random sample from the humeral shaft revealed aggregates of macrophages containing "thorotrast," but the quantities were less than in the liver, spleen, and lymph nodes. The haemopoietic cells in the immediate vicinity showed no abnormality (Fig. 11).

Lungs.—Sections confirmed the presence of blood-borne metastases and of terminal bronchopneumonia. No "thorotrast" was observed.

Distribution and Radioactivity of Thorotrast

Radiography of the liver and spleen after death showed abundant radio-opaque material in each. In the liver this was in the form of a fine tracery, presumably corresponding to the lymphatic pathways in the portal canals; concentration in the spleen was denser and coarser (Fig. 12).

Autoradiographic Studies.—Analyses of the amount and kind of radioactive substances present and the distribution of radiation dose were kindly conducted by Professor Rotblat and Dr. Ward, of the Physics Department at St. Bartholomew's Hospital Medical College, using α-track autoradiographs. Sections of liver, spleen, lymph node, and bone marrow were cut at 5 μ and coated with a layer of C2 nuclear research emulsion in liquid form (Rotblat and Ward, 1956a). Observations were made of the lengths of the α-particle tracks, of the numbers of tracks from unit volume of the sections recorded in unit time, and of the distribution and size of the "thorotrast" aggregates (Fig. 13). Over 14,000 tracks were studied, the method of analysis being that described by Rotblat and Ward (1956b) and Rotblat (1957). The average tissue dosage is given in Table III, the calculations

TABLE III
AVERAGE TISSUE DOSAGES

Tissue	Average Tissue Dosage (rads per wk.)	Dose from Largest Aggregates (rads per wk.)	Dose from Groups of Aggregates (rads per wk.)	Cumulative Average Dose (rads)
Liver	0·062	8·3	3·2–7·7	1,250
Spleen.. ..	0·51	12·4		3,600
Bone marrow	0·32	2·2	3·5	
Lymph node ..	2·2	31·0		

being based on the assumption that all the energy released as α-particles was dissipated uniformly throughout the tissue, i.e., it does not allow for variations in the dose in different parts of the organ. The effect of aggregation in the various tissues is discussed below.

Liver.—The aggregates were grouped in small areas of the tissue which appeared to be unrelated to the invading cancer tissue. From a study of seven such groups it was found that 6% of the tissue was receiving a dose varying from 3 to 8 rads per week. As well as these groups, there were a number of single small aggregates scattered throughout the tissue, which would produce a small approximately uniform dose of 0.00121 rads per week. The largest single aggregate in the tissue examined was 145 microns in radius and the dose in the surrounding 50 microns was 8.3 rads per week.

Spleen.—Almost all the activity was concentrated in the malpighian bodies, which were regarded as spheres of radioactive material of radii varying from 0.1 to 1.65 mm. These deposits would irradiate the surrounding layer of tissue 0.05 mm. thick with a dose of 8.0 to 12.4 rads per week. Within the

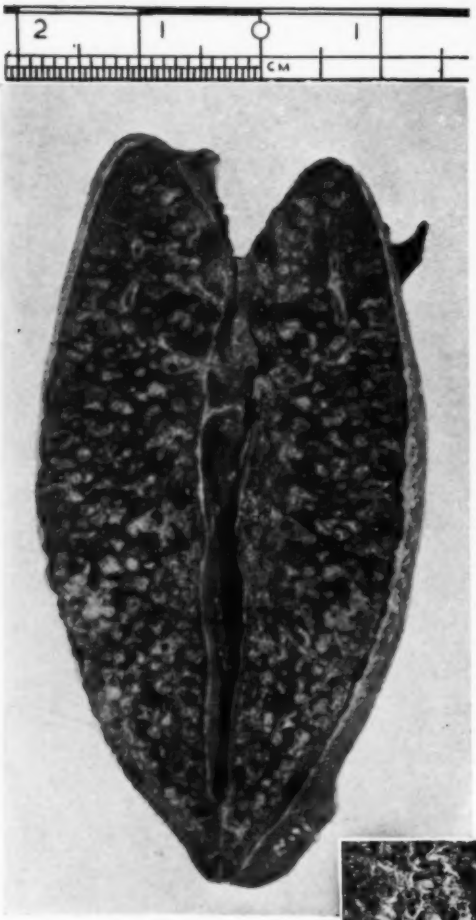

FIG. 4

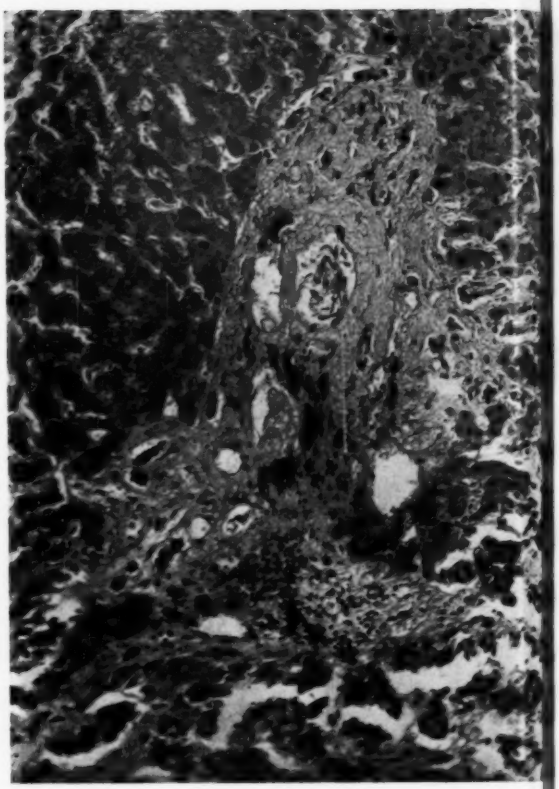

FIG. 5

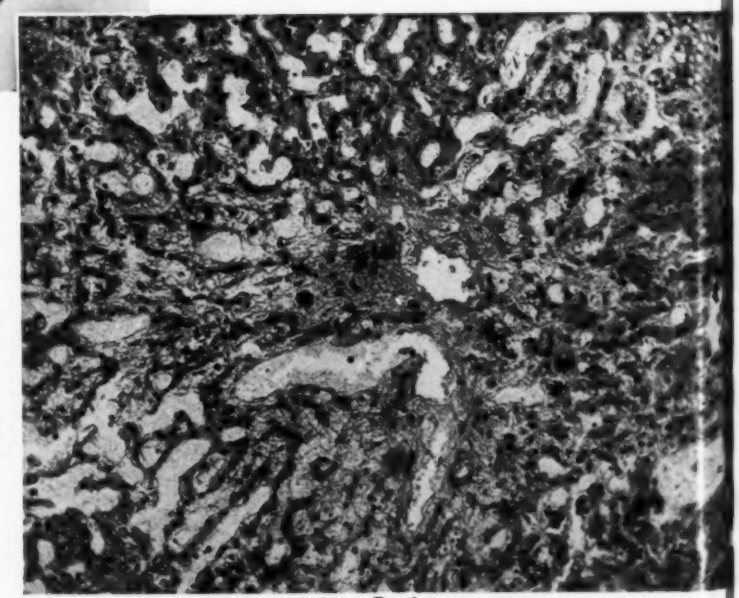

FIG. 6

FIG. 4.—Spleen showing yellowish deposits of "thorotrast" at site of malpighian bodies.

FIG. 5.—Photomicrograph of liver showing fibrous increase in portal canals related to "thorotrast" deposits. Haematoxylin-eosin × 90.

FIG. 6.—Fibrous increase round centrilobular vein related to "thorotrast" deposits. Haematoxylin-eosin × 50.

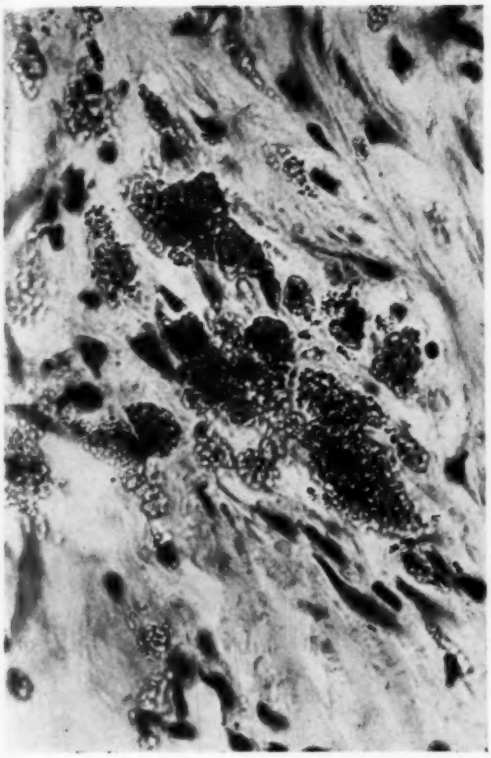

FIG. 7.—Macrophages in portal canals containing "thorotrast." Haematoxylin-eosin × 500.

FIG. 8.—Low-power view of spleen showing discrete masses of "thorotrast." Haematoxylin-eosin × 8.

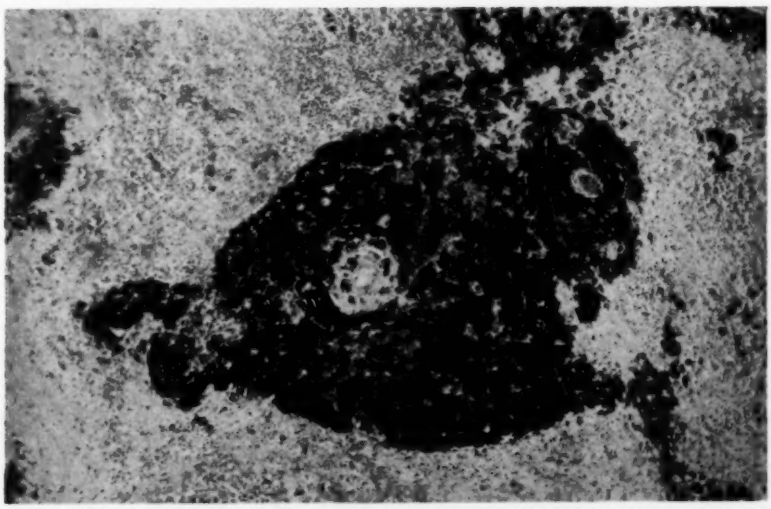

FIG. 9.—Higher magnification to show "thorotrast" deposited round penicillar artery with depletion of lymphoid tissue. Haematoxylin-eosin × 45.

FIG. 10

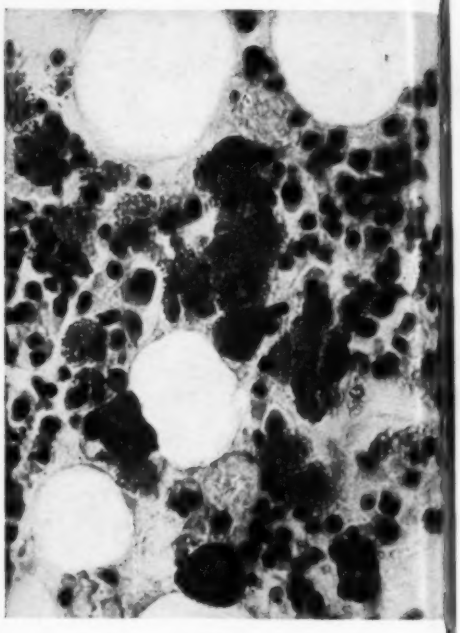

FIG. 11

FIG. 10.—Lymph node showing fibrous replacement of lymphoid tissue. Intracellular "thorotrast" near hilum. Haematoxylin-eosin × 40.

FIG. 11.—Bone marrow showing "thorotrast" in macrophages. Haematoxylin-eosin × 300.

FIG. 12.—Radiograph of liver and spleen after removal from the body (natural size).

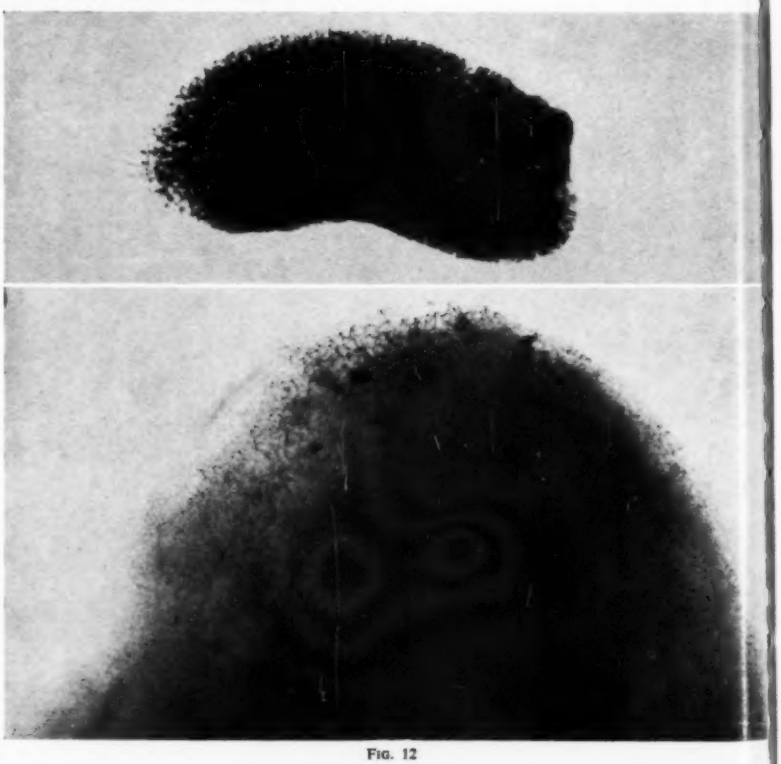

FIG. 12

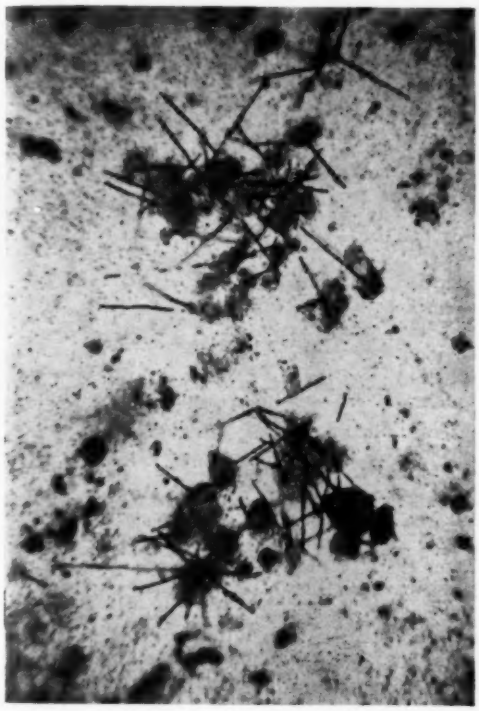

FIG. 13.—Photomicrograph of "thorotrast" aggregates in liver, showing α-tracks. (By courtesy of Professor J. Rotblat.)

malpighian bodies, the "thorotrast" was concentrated in smaller aggregates about 0.005 mm. in radius packed more or less closely together. In the less dense regions the tissue spaces were subject to a dose of 6.8 rads per week.

Lymph Node.—All cells were irradiated with a dose of at least 0.32 rads per week with many cells receiving doses of 19 to 31 rads per week from large aggregates 0.09–1.31 mm. in radius.

Bone Marrow.—All cells were irradiated with a dose of 0.17 rads per week with a few spots of higher dosage up to 3.5 rads per week.

The activity observed in the tissue sections indicated that a considerable proportion of the soluble daughter isotopes of thorium were being removed from the organs during life, the percentage of the total α-particle activity retained being only about 30% in the spleen, liver, and bone marrow and 50% in the lymph node. A rough estimate of the thorium content of the spleen and liver, obtained from the specific α-particle activity of the tissues, was 3.5 g. in each organ (Table IV), suggesting that the total volume of "thorotrast" injected into the patient was of the order of

TABLE IV
ESTIMATED THORIUM CONTENT OF SPLEEN AND LIVER

Tissue	Th Content (mg. per g.)	Mass of Organ (g.)	Total Th Content (g.)	Equivalent Volume of Thorotrast (ml.)
Liver	1·57	2,240	3·52	18·5
Spleen.. ..	122	28·4	3·47	18·3
Bone marrow	1·10	—	—	—
Lymph node ..	175	—	—	—

40 ml. In all tissues the "thorotrast" was concentrated in aggregates of varying size, so that the radiation dose was composed of a small, approximately uniform dose from small aggregates, with foci of more intense dosage in the tissue surrounding large aggregates or groups of aggregates. However, it would seem from experimental work that the thorium was initially much more uniformly distributed in the tissues and that aggregation occurred progressively during the following years. This process affects the cumulative dose to the cells in the tissues.

Assuming that the patient was injected with 40 ml. "thorotrast," the cumulative dose over 25 years was estimated to be in the order of 3,600 rads in the spleen and 1,250 rads in the liver.

Discussion

The introduction of "thorotrast" into human tissues provides a special opportunity for obtaining data on the effects of prolonged irradiation. The standard dosage of 75 ml. employed in hepatolienography involves introducing into the body some 15 g. of thorium and its derivatives. The thorium itself is retained in the reticuloendothelial system, but a number of its daughter isotopes, differing chemically from thorium, e.g., 228 Ra (MsThl), 224 Ra (ThX) and 212 Pb (ThB), are excreted from the body during the first few months (Rotblat and Ward, 1956b). These can only be distinguished from thorium (232 Th) by special observations on the type and energy of the radiation emitted. Since the variety and proportions of these isotopes in "thorotrast" vary with the method and length of time taken over the purification stages in its manufacture, and also on the time lapse between preparation and injection, up to 50% of the initial detectable radioactivity may be in the form of isotopes which are excreted, or their immediate precursors. As a result there may be a considerable fall in the irradiation being received by the body during the first six to 12 months after injection. This phenomenon may account for some of the differences in the biological fate of injected "thorotrast" described in the literature (Wichmann and Fricke, 1932;

Tripoli and Haam, 1932 ; Shute and Davis, 1933 ; Stenstrom, 1941 ; Schwaiger, Maier-Leibnitz, and Schmeiser, 1949).

The radioactivity of some of these daughter isotopes is greater than that of the same mass of thorium, and as a result there is considerable variation with time in the total α particle energy dissipated in the body. This energy is dissipated in the cells in the immediate vicinity of the isotope, i.e., the reticulo-endothelial system, since α particles have a very short range. If the only isotope in 75 ml. of " thorotrast " were thorium (232 Th), such energy would be in the order of 0.4 ergs/sec. initially, and theoretically be capable of increasing by a factor of 10 if the daughter isotopes formed accumulated in the tissues (Taft, 1937b ; Reeves and Morgan, 1937 ; Rundo, 1956). Rundo found 224 Ra (ThX) and 212 Pb (ThB) in human blood many years after the injection of " thorotrast," an observation at variance with the claims of Looney, Arnold, Levi, and Jee (1955) that after 20 years there is very little further loss of radioactivity from the isotopes remaining in the body.

The radioactivity of the tissues increases after death, indicating that the " fixed " isotopes are not in equilibrium with their daughter products, presumably because the latter are soluble and are continually being eluted during life. This phenomenon, which depends on the rate of extracellular fluid exchange around the " fixed " isotopes in the aggregate (and therefore to some extent on the degree of fibrosis around them), may explain the lack of consistency in the proportions of the daughter isotopes of thorium which Rundo observed in the tissues (a) between different patients ; (b) between the various organs of the same patient ; and (c) between the different parts of the same organ.

The relation of fibrosis to the " thorotrast " deposits has been commented on by various authors. The appearances in the lymph nodes and spleen suggest that the degree of fibrosis may be related to the concentration of the drug. Certainly dense scarring may follow leakage of " thorotrast " into the tissues surrounding veins (Yater and Whitmore, 1938 ; Amory and Bunch, 1948), and in one case sarcomatous change supervened (Plenge and Krückemeyer, 1954).

The manner in which the fibrosis is produced is unsettled. Naegeli and Lauche (1933) thought that a sufficient concentration of " thorotrast " could cause cell death, followed by fibrosis, but it is not clear whether this is brought about by the physical effects of a foreign body, the chemical properties of the drug (cf. silicosis), or the radioactivity of thorium and/or its derivatives. Rigler *et al.* (1935) regarded the fibrosis as a toxic effect rather than due to irradiation. Thomas *et al.* (1951) held the opposite view.

We incline to the belief that the fibrosis is a low-grade inflammatory response to repeated necrosis of cells within the range of the radioactive deposits. The α particles have a mean range of about 0.05 mm., and a very high specific ionization ; therefore they are biologically most dangerous, since the amount of tissue injury is related to the specific ionization (Gray, 1953).

Whatever the cause of the fibrosis in the liver, there is no convincing evidence that " thorotrast " can induce cirrhosis of the Laennec type, as has been suggested by Cassel, Ruffin, Reeves, and Stoddard (1951) and Jonsell and Lindgren (1944). In experimental animals the drug causes an increase in connective tissue, but such intracellular changes that occur do not cause disturbance of the lobular architecture (Naegeli and Lauche, 1933 ; Tripoli, 1934) and the same appears to hold for the human liver (Jacobson and Rosenbaum, 1938 ; Groskopff, Bolck, and Büll, 1951 ; Berenbaum and Birch, 1953).

The point is of some interest, since, according to Moore (1951), about 90% of all liver-cell carcinomas and 50% of all bile-duct carcinomas occur in livers with cirrhosis. In these cases of cirrhosis it is reasonable to suppose that cellular multiplication in the surviving lobules undergoing compensatory hyperplasia is a greater factor in carcinogenesis than the mere presence of fibrous tissue. It is important to note, therefore, that the fibrous tissue increase in the portal canals and round the central veins, related to the deposition of " thorotrast " and described by many authors, is unaccompanied by disturbance of lobular architecture and unlikely to be in itself a factor in subsequent carcinogenesis.

Malignant growths of the liver following the intravascular administration of " thorotrast " fall into two broad groups: the haemangio-endotheliomata or endothelial cell sarcomata on the one hand, and the primary carcinomata of liver-cell or bile-duct origin on the other. Horta (1956) thinks that all the genuine cases have belonged to the first group, but this is to ignore the claims of Matthes (1954), Heitmann (1954), Grossiord *et al.* (1956), Roberts and Carlson (1956), and our own case. It may be of interest to record that both types of tumour have been reproduced experimentally in animals by injecting " thorotrast " (Zeitlhofer and Speiser, 1954 ; Guimaraes *et al.*, 1955).

It is still a matter of opinion whether the number of recorded cases is enough to justify the disuse of "thorotrast," if indeed the tumours are produced by irradiation at all. Bauer (1948) showed that the radiation given off by "thorotrast" is 6 r per day, which by current estimates is about 140 times the amount usually cited as the maximum daily permissible dose in regulations for protection against *x* rays. Yet Thomas *et al.*, writing in 1951, considered that the five malignancies recorded up to that time did not indicate the carcinogenic properties of "thorotrast" in man; and Looney (1954, 1955) considers that no significant number of clinical disorders have resulted from its use, based on a follow-up of 4,800 individuals.

The number of case records in which there has been good reason to ascribe malignancy to "thorotrast" has steadily mounted, and it is our view that this trend is likely to continue during the next few years. Furthermore, it is certain that a number of cases have gone unrecorded, where there has been no necropsy, or where the pathologist has not recognized the deposits of "thorotrast" or connected them with a new growth.

In our view primary growths arising in close proximity to deposits of "thorotrast," in the absence of other stimuli to neoplasia such as cirrhosis and chronic inflammation, can reasonably be regarded as irradiation phenomena. Until this is generally accepted it is worth while to record such cases in the medical literature.

Summary

(1) A case of primary liver-cell carcinoma occurring 24 years after the intravenous injection of "thorotrast" is described, together with the methods of assessing the residual radioactivity in the organs after death.

(2) Similar case records are critically examined.

(3) It is concluded that malignancy arising at the site of "thorotrast" deposit is likely, if there are no other predisposing factors, to be an irradiation phenomenon.

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Addendum

Since this article was submitted, several cases of malignant tumours following the use of thorotrast have been published: Boemke (1956) described an epithelioma of the renal pelvis after retrograde pyelography; Batzenschlager and Wilhelm (1957) reported primary carcinoma of the liver 11 years after arteriography of a limb. Unfortunately there was no proper necropsy, and the possibility of a primary elsewhere was not eliminated. Federlin and Scior (1957) record a liver cell carcinoma 13 years after cerebral angiography, but the patient also had a rectal carcinoma. They also attribute an ovarian carcinoma to a salpingography 23 years earlier. Other cases of liver tumours have been reported by Caroli, Etévé, and Platteborse (1956) and Fallot (1956), but we have not been able to obtain the journal in which they appear.

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J. clin. Path. (1958), **11**, 19.

FOAM CELLS IN THE STROMA OF CARCINOMA OF THE BODY OF THE UTERUS AND UTERINE CERVICAL POLYPI

BY

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Dubs (1923) and Nunes (1945) each described a case of adenocarcinoma of the body of the uterus in which foam cells were present in the stroma of the neoplasm; these are the only instances recorded in the literature. Foam cells do not seem to have been previously reported as occurring in the stroma of uterine cervical polypi. Two cases of this condition have been observed recently in cervical polypi and prompted an investigation of the frequency of this phenomenon in other gynaecological material. The results of this search are shown in Table I.

TABLE I
FOAM CELLS IN MATERIAL EXAMINED

Condition	No. of Cases Examined	No. of Cases with Foam Cells in Stroma
Cervical polypi	400	2
Endometrial ,, ...	200	0
,, carcinoma	150	16
Normal endometrium		
(*a*) Proliferative	100	0
(*b*) Secretory	100	0
(*c*) Senile	100	0
Endometrium in metropathia		
(*a*) Cystic glandular hyperplasia	50	0
(*b*) Less marked hyperplasia ..	50	0

Histological Description

Adenocarcinoma of Body of Uterus.—Foam cells were found in 11% of the endometrial carcinomata. All but one of these tumours were well-differentiated adenocarcinomata. The remaining neoplasm showed moderate differentiation, but the foam cells were restricted to those areas in which there was an attempt to form acini. In general, the foam cells occurred in the superficial portions of the growth, where they formed plaques of variable size between the columns of neoplastic cells (Fig. 1). These foam cells usually were packed close together, but in some instances were separated from one another by a small amount of blood or a few connective tissue cells. In certain areas the cells lay in very close proximity to the malignant cells, and because of this their differentiation from the neoplastic cells and from the clear cells of the endometrium was sometimes difficult in sections stained by haematoxylin and eosin (Fig. 2). By using Masson's trichrome stain, the foam cells could be distinguished because their granules stained greenish brown, the granules of the tumour cells stained dark brown, and there were no granules in the clear cells of the endometrium. With this stain it was clear that the foam cells

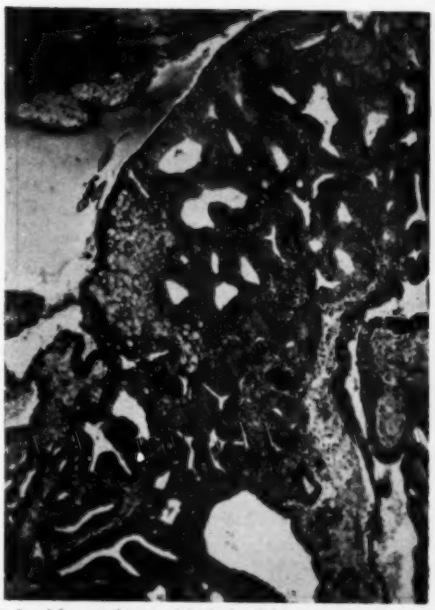

FIG. 1.—Adenocarcinoma of the body of the uterus showing plaques of foam cells between neoplastic cells, × 60.

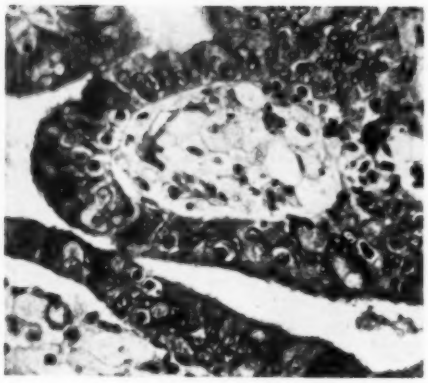

FIG. 2

FIG. 3

FIG. 4

FIG. 5

FIG. 2.—Same tissue as Fig. 1 showing a plaque of foam cells in close proximity to neoplastic cells, × 250.

FIG. 3.—Curettings showing foam cells in stoma of cervical polyp, × 20.

FIG. 4.—Same tissue as previous figure showing details of foam cells in closely packed clearly demarcated masses, × 90.

FIG. 5.—Same tissue as Fig. 4 showing foam cells separated from each other by blood, × 90.

sometimes indented the overlying malignant cells but never penetrated between them.

Cervical Polypi.—In the first case most of the core of the polyp was composed of foam cells, which gave the polyp an unusual and arresting histological picture (Fig. 3). The cells were closely packed together to form clearly demarcated masses (Fig. 4) or were separated from one another by a variable amount of blood (Fig. 5). Fibrous tissue or areas of haemorrhage separated the foam cells from the epithelium, which covered the external surface of the polyp. In the second case, the foam cells were not as numerous as in the first one and did not form such a prominent histological feature. Most of them lay in a dilated glandular space situated within the core of the polyp. Haemorrhage had taken place into this space and the foam cells were scattered in small groups throughout it. In one place the continuity of the lining epithelium of the gland had been broken, and beneath this the surrounding connective tissue stroma contained a number of foam cells (Fig. 7).

Minute Histological Structure of the Foam Cells

The detailed histological features of the foam cells were the same in the cervical polypi and the endometrial carcinomata (Fig. 6). The cells were large and irregular in shape; their nuclei were eccentric in position and had a variable appearance. Some of them were round or oval; they possessed a prominent nucleolus and the chromatin was dispersed evenly in fine granules. In other instances, the nuclei were irregular and showed a variable degree of pyknosis with condensation of their chromatin. In sections stained by haematoxylin and eosin, the cytoplasm had a foamy appearance due to the presence of minute pink granules. The granules stained greenish brown with Masson's trichrome stain, and pink with the periodic-acid-Schiff reaction. Best's carmine stain and the periodic-acid-Schiff reaction before and after treatment with diastase did not show the presence of glycogen. Paraffin sections stained with osmic acid and frozen sections stained with a mixture of Sudan III and IV revealed the presence of black or orange granules respectively in the cytoplasm of the foam cells. Refractile bodies were not seen when paraffin and frozen sections were examined by polaroid screens. Sections stained by Perles' method gave a negative result.

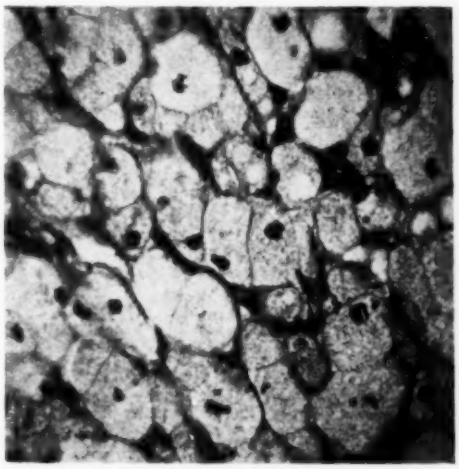

FIG. 6.—Same tissue as Fig. 4 showing morphological features of foam cells, × 400.

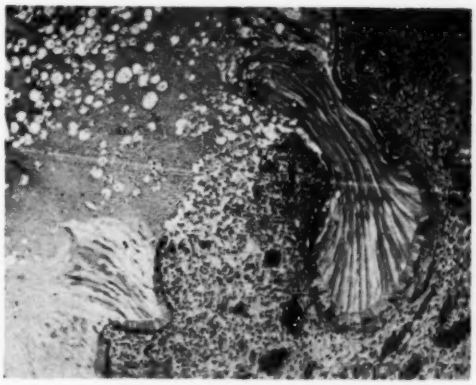

FIG. 7.—Cervical polyp showing a break of continuity of the epithelium of a glandular space in the polyp. Blood and foam cells are present in this area, × 60.

Discussion

Only two cases of endometrial carcinoma containing foam cells have been recorded (Dubs, 1923; Nunes, 1945). The occurrence, therefore, of foam cells in 11% of endometrial carcinomata is unexpectedly high, but their appearance in cervical polypi is a rare phenomenon.

Foam cells have been described in a variety of tumours, for example, in prostatic carcinoma (Kinoshita, 1920), dermoid cysts (Stewart, 1924), tendon sheath tumours (Garrett, 1924), renal carcinoma (Nathan, 1933), specific nerve sheath tumours (Stout, 1935), sclerosing haemangioma of the brain (Bailey and Ford, 1942), sclerosing

haemangioma (Dawson, 1948), non-osteogenic fibroma of bone (Lichtenstein, 1952), and in lipoma synovioma and renal adenoma (Evans, 1956). Dawson (1948) considered that foam cells arose as the result of haemorrhage into the tumour. As Perles' reaction is negative, this mechanism will not explain the occurrence of these cells in the cervical polypi or endometrial carcinomata.

Xanthoma cells similar to the foam cells in the cervical polypi are found in the systemic lipidoses, but in these diseases the histological appearance is different, as at the site of the deposition of fat there is an increased fibroblastic activity often associated with giant cell formation. In the first cervical polyp the blood lipids were normal and lesions were not found in other parts of the body.

Aaron and Levine (1954) described an endometrial oil granuloma, which followed a hysterosalpingography. In the cases described in this paper, fatty substances had not been applied locally to the genital tract.

As Gillman (1941) found an increase of stromal fat in metropathia and Black, Heyns, and Gillman (1941) showed that the administration of oestrogens to a castrated woman caused lipid to accumulate in the stromal cells, the foam cells in the cervical polypi and endometrial carcinomata might have been evoked by the action of oestrogens. The patient with the cervical polyp, which contained a large number of foam cells, had taken large amounts of oestrogens, but in the other cases there was no history of the administration of oestrogens. Foam cells were not found in the normal endometrium or in the endometrium of cases of metropathia. It is considered, therefore, that oestrogens are not influential in the production of foam cells.

Wegelin (1947) noted the similarity in appearance of the cells of a granular cell myoblastoma with those found in Gaucher's disease. Sadler and Dockerty (1951) reported one, which arose in the vulva, and Powell (1946) recorded another, in which the ovary contained a secondary deposit. It is unlikely that the first cervical polyp is a granular cell myoblastoma, as the foam cells are situated in the stroma and have a different morphological arrangement to that seen in this tumour.

A common aetiological factor may be responsible for the production of the endometrial clear cells and the foam cells. Feyrter (1949 and 1952) considered that the clear cells formed an endocrine system. Farkas (1952) and Jakobovits (1955) believed that their presence was due to the action of oestrogens. Rotter and Eigner (1951) stated that these cells were degenerative in origin. If the latter concept is accepted both the clear cells and foam cells could be the result of a degenerative process, but if one acknowledges the other opinions the simultaneous appearance of these cells for reasons already stated will be due to different aetiological factors.

It is considered from the above discussion that the cases described in this paper are examples of foam cells occurring in tumours. There is insufficient evidence to explain the processes which give rise to these cells.

Summary

Foam cells were found in the stroma of only two cervical polypi out of a series of 400 cases.

In a series of 150 endometrial carcinomata foam cells were present in the stroma of 16 cases.

Foam cells were not demonstrated in a series of 200 endometrial polypi, in the normal endometrium, or in the endometrium of cases of metropathia.

The various aetiological factors which could be concerned in the production of foam cells in the cervical polypi and the endometrial carcinomata have been discussed. It was considered that there was insufficient knowledge to explain the processes which are responsible for the development of these cells.

I wish to thank the surgeons of the Women's Hospital, Liverpool, for most of the material, and Mr. Corbett and Dr. Pantin, who provided me with material from an endometrial carcinoma which contained foam cells.

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NON-SILICIOUS, DOUBLY REFRACTILE CRYSTALS IN THE GIANT CELLS OF TUBERCULOSIS

BY

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The diagnosis of silicious or talc granuloma has often been made upon histological evidence unsupported by any chemical tests. Such a diagnosis would appear to be confirmed by finding non-caseating "tubercle" follicles and doubly refractile crystalline particles in or near multinucleate giant cells, with a negative staining reaction for acid-fast bacilli.

Five cases of silicious granuloma in the Fallopian tube were described by Roberts (1947), appendicectomy having been performed previously in each of these cases, but in only one was the diagnosis proved by chemical tests.

Smith (1945) recorded an unusual case of carcinoma of the body of the uterus together with tuberculosis of the endometrium and Fallopian tubes. After the publication of the article by Roberts (1947), Smith (1948) re-examined his sections and found doubly refractile bodies within giant cells, which convinced him that the lesions were silicious and not tuberculous. No chemical tests were performed to confirm or refute this amended diagnosis.

Potts (1951) reported two cases of tuberculous salpingitis which showed typical caseating tubercle follicles and multinucleate giant cells. Sections from both the cases contained strongly doubly refractile crystalline particles within giant cells, which morphologically resembled talc. When subjected to an acid-solubility test and micro-incineration, the crystals were shown not to be silicious. Although no acid-fast bacilli were discovered when similar sections were specially stained, the lesions were considered to be tuberculous and not talc granulomata.

Burne (1953) attempted to verify the association of laminated (Schaumann) bodies and doubly refractile bodies with tuberculous salpingitis. Eleven of the 42 cases reviewed showed acid-soluble, i.e., non-silicious, birefringent crystals, mostly in multinucleate giant cells. The absence of silica was confirmed in two cases by micro-incineration of sections. In addition, one case of talc granuloma was discovered, silica being identified by chemical tests.

A tubal pregnancy occurring in a tuberculous uterine tube was reported by Berenbaum and Korn (1954). The tuberculous lesion contained many doubly refractile crystals within giant cells, which were shown by histochemical analysis to consist of calcium carbonate.

Pearse (1953) shows illustrations of birefringent crystalline inclusions within multinucleate giant cells in sections from a human lymph node.

This article briefly describes a case in which the histological appearance of the lesion closely simulated that of a talc granuloma, but was later proved to be tuberculous. Findings from a series of histochemical investigations carried out on Fallopian tubes and other tuberculous organs are recorded and commented upon.

Case Report

Mrs. R. was first seen in 1956 complaining of four years' infertility. A year previously infertility investigations had been carried out; at that time a tubal insufflation and curettage had, apparently, revealed no abnormality. A salpingogram was performed which showed dilated uterine tubes, both blocked at the fimbrial ends.

At operation the naked-eye appearance of the Fallopian tubes was at first sight completely normal. The fimbriae and serous coat showed no abnormality even on close inspection. However, on attempting to pass a probe through the abdominal ostia it became apparent that there were very tight strictures at the bases of the fimbriae. The width of the strictures was no wider than a thin piece of string. On making incisions through the narrow strictures it was seen that the lumen of each tube was somewhat dilated and the wall of the tube somewhat oedematous and thickened. There were no other strictures visible or palpable along the course of the tubes. A piece of tissue was removed from the site of one stricture and bilateral salpingostomy was performed.

Pathology

Portions of Fallopian tube were used for histological examination, guinea-pig inoculation, and culture for tubercle bacilli on egg media. A section of the tubal tissue stained with haematoxylin and eosin showed non-caseating tubercle follicles with multinucleate giant cells, many of the latter containing crystalline particles. These crystals were strongly doubly refractile when examined in polarized light. No tubercle bacilli were found, using both the Ziehl-Neelsen and auramine-rhodamine-phenol techniques.

From these findings a diagnosis of talc granuloma was made. Histochemical tests were then performed in an attempt to confirm the silicious nature of the anisotropic crystals. The results proved that the doubly refractile crystalline particles were non-silicious, and therefore not talc. The tubal lesions were shown to be tuberculous in nature by the growth of *M. tuberculosis* both on the egg media and in the inoculated guinea-pig.

Having found a large number of intracellular, doubly refractile crystals in a proved case of tuberculosis, it was decided that sections from other cases of tuberculous salpingitis should be examined to ascertain the incidence of these inclusions.

Material and Methods

Sections from the cases of tuberculous salpingitis seen in the hospital during the past 10 years were examined. Thirty such cases were reviewed, and, in addition, a case reported as "pseudo-tuberculous foreign-body reaction in the Fallopian tube due to talc" was examined, which, with the one already described, made a total of thirty-two. Besides the original slides, one or more blocks of wax-embedded tubal tissue were available from every case. The slides were cleaned with xylol to remove any wax which is itself brilliantly doubly refractile. The sections were "screened" under low power in polarized light, using a Swift polarizing microscope. It was found easiest to perform this manœuvre with the planes of the polarizer and analyser not quite fully crossed. This enabled any birefringent particles to be picked out easily, and yet gave sufficient general illumination to make it possible for the relationship of the particles to the surrounding tissues to be seen. Only those doubly refractile particles seen to lie within multinucleate giant cells were recorded as being significant for this investigation. Only by using this strict criterion was it possible to exclude material not derived from the specimen, for many doubly refractile particles were seen other than those in giant cells, possibly introduced during the processing of the sections.

Six new sections were cut from each block corresponding to the sections found to contain doubly refractile inclusions. Each set of six sections was used as follows:

(1) One section was stained with haematoxylin and eosin and mounted under a coverslip in the usual way.

(2) Two sections had the wax dissolved away, were lightly stained with aqueous methylene blue, and left uncovered.

(3) The remaining three sections were kept as "spares."

The two lightly stained sections were subjected to screening in polarized light after the application of a drop of xylol and a thin coverslip. The position of any birefringent particles in multinucleate giant cells was noted on the vernier scales of the microscope. If no inclusions were found, the three spare sections were similarly stained and examined.

When birefringent particles were discovered in a section the coverslip was removed, and, after evaporation of the xylol, the section itself covered with dilute hydrochloric acid (N/1). After 30 minutes the acid was gently rinsed off with distilled water and the section allowed to dry. The areas which had contained birefringent particles were then re-examined in polarized light to ascertain whether the inclusions had been dissolved.

The second slide containing intracellular crystals was subjected to micro-incineration, using the technique described by Irwin (1934). A small electrically heated muffle-furnace was utilized, the slides being placed on a stainless steel tray and gradually heated to 600° C. (dull red heat), this temperature being maintained for one hour. After cooling, the areas containing birefringent particles were re-examined, first in polarized light and then by reflected light. The appearance of the incinerated inclusions was noted and the ash then treated with dilute hydrochloric acid (N/1) for 30 minutes. If the inclusion ash had not been dissolved by the dilute acid, the effect of concentrated hydrochloric acid was also investigated.

To act as a known positive control, a sample of surgical talc was fixed in formol saline, and embedded in wax in exactly the manner used for tissues. Six sections were prepared, two of which were subjected to the tests described. Material containing silica (talc in this instance) was found to be insoluble in dilute and concentrated acid, both before and after micro-incineration, the latter process altering neither the appearance of the crystals nor their birefringence.

Results

Seventeen of the cases investigated revealed multinucleate giant cells containing birefringent particles, the two "talc granuloma" cases being included among these. The number of separate inclusions encountered in the "positive" slides ranged from one to more than 100. In every case the birefringent particles in the giant cells presented either as single, irregularly shaped, but rather rounded crystals, or as round or oval aggregates of similar material. The size of the individual particles was variable, reaching a maxi-

mum of about 40 μ. By transmitted light the crystals appeared colourless or a very pale yellow. Viewed in polarized light, with the planes crossed, they became brilliantly doubly refractile, many of the larger crystals exhibiting a play of colours (Figs. 1 and 2).

On comparison, the crystals of the talc control were as variable in size, but morphologically were much less rounded. Talc was found also to be much less birefringent, many of the larger crystals being doubly refractile only at the edges.

All the birefringent inclusions were found to be soluble in dilute hydrochloric acid, leaving round or oval deficiencies in the giant cells corresponding to the size and shape of the crystals dissolved away.

Micro-incineration converted the birefringent crystals into white amorphous material, the particles of which were approximately the size of the original crystals, showing much reduced or absent birefringency. Most of these altered particles dissolved rapidly in dilute hydrochloric acid, but a few required concentrated acid.

From these investigations it was concluded that the material in the multinucleate giant cells was not silicious. This included the material in both the cases previously described as being talc granulomata.

Relationship of Inclusions to Caseation.—The 32 cases were divided into those showing and those not showing caseation. Doubly refractile inclusions were found in 8 of the 11 with no caseation and in only 9 of the 21 with caseation.

Similarly, when the frequency of the inclusions was investigated in the 17 positive cases, it was usually the non-caseating lesion which contained the large number of birefringent inclusions.

Intracellular Birefringent Crystals in Other Organs

As over half of the tuberculous Fallopian tubes were found to contain doubly refractile crystals, an attempt was made to discover whether this finding was common to tuberculous lesions in other tissues. Sections from 10 cases

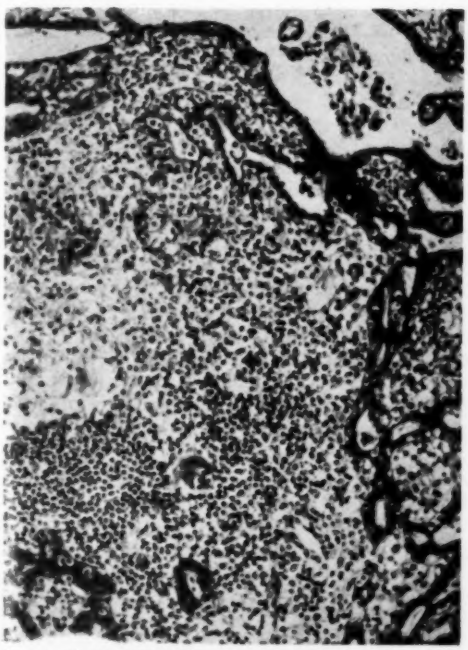

FIG. 1.—Section of Fallopian tube photographed in ordinary light showing a non-caseating tubercle follicle with multinucleate giant cells. (Haematoxylin and eosin, ×130.)

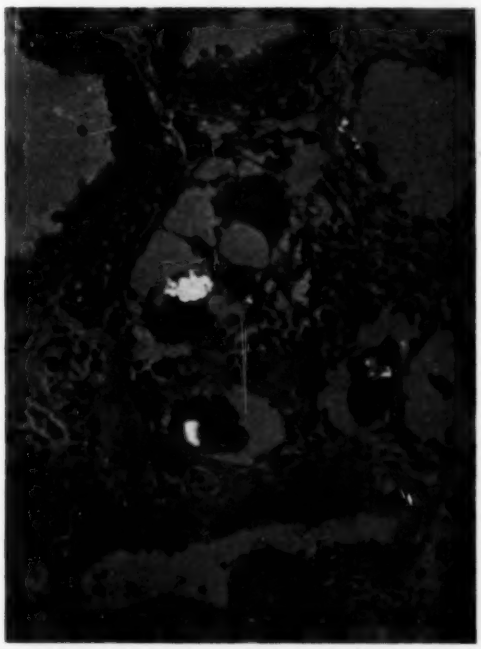

FIG. 2.—Part of same section as Fig. 1 photographed in polarized light with planes not quite fully crossed. Four giant cells contain doubly refractile inclusions. (Haematoxylin and eosin, ×200.)

each of tuberculous lymph nodes, kidney, and epididymis were studied. Lung tissue was excluded because of the possibility of exogenous silica being present. The methods used for screening, followed by chemical tests, were similar to those used for the Fallopian tubes, with the following results:

Lymph nodes	..	10 cases studied, 3 contained crystals					
Kidney	.. ..	10	,,	,,	8	,,	,,
Epididymis	..	10	,,	,,	6	,,	,,

This means that 17 (57%) of the 30 cases examined showed birefringent crystalline inclusions in giant cells almost identical with those found in the Fallopian tubes. They showed the same variation in number, but were usually smaller and more widely scattered through the sections (Fig. 3).

Acid-solubility tests and micro-incineration showed that the crystals behaved exactly as did those from the tubes, and therefore were also non-silicious.

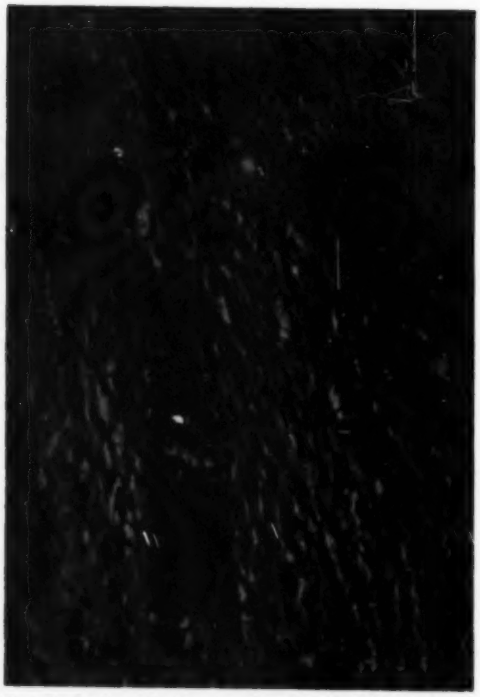

FIG. 3.—Section of tuberculous lymph node, photographed in polarized light. Two giant cells contain small birefringent inclusions. (Haematoxylin and eosin, ×200.)

Chemical Composition of the Crystals

A limited histochemical analysis was attempted using only sections from a case of tuberculous salpingitis showing numerous inclusions.

The crystals gave a strongly positive reaction to von Kossa's test, thus demonstrating them to be either phosphate or carbonate. When glacial acetic acid was applied to the inclusions by running it under a coverslip, the crystals were seen to dissolve without any bubble (CO_2) formation. The crystalline material was therefore, in all probability, a phosphate.

Pearse (1953) states that nearly all deposits of insoluble carbonate or phosphate in animal tissue are calcium salts. It can be presumed therefore that the material investigated was a calcium phosphate. Some confirmation of this was obtained by subjecting a preparation of known calcium phosphate crystals to all the tests described here, with results identical with those obtained with the inclusions.

Comment

Burne (1953) investigated 41 cases of tuberculous salpingitis and found Schaumann bodies in 29% and doubly refractile crystalline inclusions in 27% of them. In the present series similar crystals were found within multinucleate giant cells in 55% of all the cases studied (a total of 62). This result surely demonstrates that doubly refractile crystals within multinucleate giant cells occur very frequently, not only in tuberculous salpingitis but also in tuberculous lesions of other organs.

Potts (1951) found that birefringent crystalline inclusions were scanty or absent when caseation was present. The present findings seem to confirm this. This increased frequency in non-caseating tuberculous lesions has, on occasions, possibly led to a mistaken diagnosis of talc granuloma being made. If, therefore, the diagnosis of silicious granuloma is to be made with certainty, the quick and easily performed acid-solubility test should be carried out on the crystals. This is not put forward as an original suggestion, for Potts made the observation in his paper, but it is reiterated and emphasized here because it seems to be of such importance.

Summary

A case of tuberculous salpingitis is described on which a diagnosis of talc granuloma had been made originally.

Investigation of 32 cases of tuberculous salpingitis showed sections of the lesions from 17 of them

to contain birefringent, non-silicious crystalline inclusions within multinucleate giant cells.

A further 30 cases with tuberculosis of other organs (excluding lung) similarly investigated showed sections from 17 of them to contain similar crystalline inclusions.

The birefringent crystals in giant cells were found to be more common when caseation was absent, confirming the findings of other workers, and in one case were shown to consist probably of calcium phosphate.

The necessity of performing a simple acid-solubility test on the birefringent inclusions before making a diagnosis of silicious granuloma is emphasized.

We are indebted to Mr. J. H. Peel for permission to publish the case; to Professor H. A. Magnus for his advice and encouragement; and to Mr. G. Harwood for the photomicrographs and technical assistance.

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FAT EMBOLISM STUDIED IN 100 PATIENTS DYING AFTER INJURY

BY

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Historical

The occurrence of fat embolism as a complication of traumatic injury was first recognized in the latter half of the nineteenth century. Much work has since been done on the condition and the literature is peculiarly rich in references. Wilson and Salisbury (1944) estimated their number at 600, and Warthin (1913) and Groskloss (1935–36) have reviewed the extensive literature. Discussion has centred around four main points: (1) the type of illness or injury commonly associated with fat embolism; (2) the origins and mode of release of the emboli; (3) the frequency; and (4) the significance of fat embolism.

The first two points have been thoroughly investigated. Various authors have published reports of the frequency of fat embolism in necropsy material (Olbrycht, 1922; Vance, 1931; Whiteley, 1954) and have concluded that, while occasional pulmonary fat emboli may be found in a variety of conditions and a slight degree of fat embolism is commonly found after burns, fat emboli in significant numbers are only found after injury or illness characterized by fits or convulsions. Serious and fatal degrees of fat embolism have been reported after slight injuries (Ziemke, 1922), but are commonly found only in patients with severe injuries, particularly those with fractures of long bones. In the present investigation these findings have been confirmed in a small series.

Aetiology and Pathogenesis

Fat emboli are believed to originate in the marrow of bones injured by fracture, or rarely by general skeletal shaking without fracture ("*Erschütterung*") by rupture of marrow fat cells. Reports of fat embolism in patients with widespread bruising of the subcutaneous fat have been made (Scully, 1956), but in these patients there must have been a severe degree of general skeletal shaking and the source of the emboli is still in question. When free fat is released into the marrow cavity of a fractured bone, increased medullary pressure forces it into the venous sinuses which are held open by their attachments to the bony trabeculae. The fat traverses the right side of the heart to impact in the pulmonary vessels, and some may penetrate to the systemic circulation where it again lodges as emboli.

Other views have been advanced as to the origin of the fat, notably that it results from a breakdown of the physiological fat emulsion of the plasma, either from the stimulus of metabolites released by the injury, or "triggered off" by a small amount of free circulating fat (Lehman and Moore, 1927). The experimental evidence advanced in support of this theory is not convincing, and it has not gained wide credence. One of the stimuli to the production of this theory was the statement that there is not enough fat in the marrow of one femur to produce death by fat embolism, even if it all be released as emboli. This has been disproved by Glas, Grekin, Davis, and Musselman (1956) and by Peltier (1956).

Two main clinical types of fat embolism have been described.

Pulmonary Fat Embolism.—This is said to be characterized by dyspnoea, cyanosis, sputum, which may be copious and frothy or slightly blood-stained, and increasing respiratory embarrassment leading in some cases to death. Necropsy examination is said to show characteristic changes in the lungs (Robb-Smith, 1941). They are described as being heavy and voluminous, firm but not solid, with a marbled appearance of the visceral pleura due to alternating areas of haemorrhage and emphysema. Tardieu spots may be present beneath the visceral pleura. Histological examination shows capillary congestion, intra-alveolar haemorrhage, and oedema with zones of emphysema.

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Cerebral Fat Embolism.—The symptoms of cerebral fat embolism may follow those of pulmonary fat embolism or occur without warning. A delay of 24 to 72 hours after injury before the development of symptoms is described as characteristic, but the examination of the records of published cases and personal experience shows the delay to be less than commonly supposed. The patient is generally conscious after injury and loses consciousness later. This loss of consciousness may be sudden or gradual, and may be preceded by a period of restlessness or even mania which has on occasions been diagnosed as delirium tremens. It may also manifest itself as a failure to recover consciousness after anaesthesia. Another initial sign is incontinence in a previously continent patient. Whilst unconscious the patient may show various signs of disturbance of the central nervous system such as paralyses, spasticity, loss of reflexes, or fits. A rise in temperature is often seen, and may reach 105° F. A petechial skin rash may also be present, most marked over the anterior upper thorax and shoulders and in the conjunctivae. At necropsy the macroscopic appearances are of a brain under slightly increased pressure, with congested vessels, in which section shows numerous haemorrhages, ranging in size from petechiae to that of a pea, distributed throughout the white matter of the cerebral hemispheres. Haemorrhages may also be present, but are less common, in the cerebral grey matter and in the brain-stem and cerebellum.

Material and Methods of the Present Investigation

Trauma Series.—Material consisted of clinical records, necropsy reports, and histological preparations from 100 patients who died after injury.

Control Series.—Material from 53 patients who died without suffering trauma was used. Because of the specialized nature of the hospital, a large proportion of the deaths were from burns, and of these a large number occurred in children. This is not a satisfactory control group, as burns are known to give rise to a constant though minor degree of fat embolism (Olbrycht, 1922) and fat embolism is said to be less common in children than in adults (Whiteley, 1954; Wright, 1932). In spite of these objections, there is a qualitative difference between the results in the two series.

Methods

Quantitative Estimation of Pulmonary Fat Embolism.—Various workers have defined mild, moderate, and severe pulmonary fat embolism to their own satisfaction, but it is very difficult to compare various sets of data with varying, largely subjective criteria. So far as can be ascertained, only one study has been made in which fat emboli per unit area of lung section have been counted, and this was done in experimental animals (Swank and Dugger, 1954). It seemed important that the assessment of pulmonary fat embolism should be made on as strictly a numerical basis as possible in order that comparison between different cases should be easy and accurate. Accordingly, in this investigation the numbers of fat emboli have been counted in standard areas of lung sections. Frozen sections 15 μ thick were used, stained with oil red O and light green, and in each section all the emboli in 20 randomly selected fields were counted. The magnification used was 90. Each field was 0.95 mm. in diameter and 2.985 sq. mm. in area, making the total area counted in each section 59.7 sq. mm. Fat emboli in the lungs are recorded as numbers in this, the unit, area. Emboli were counted individually; there was no difficulty with those obviously intravascular, but some did arise with fat globules in the alveoli. It was considered that where intravascular emboli were numerous, extravascular globules of 10 μ or more in diameter were in all probability extravasated emboli, and they were counted as such. Fat globules smaller than these were ignored, and in lungs with small numbers of intravascular emboli, extravascular fat, which was very small in amount in such lungs, was excluded. The proportion of extravascular to total fat was in no case high, and its inclusion or exclusion would not have altered the group into which a case fell.

Cases were classified as mild, moderate, or severe pulmonary fat embolism. Mild fat embolism included cases with from one to 20 emboli per unit area; moderate fat embolism was from 21 to 60 emboli per unit area, and severe fat embolism more than 60 emboli per unit area. The definition of mild fat embolism adopted approximates to the "mild fat embolism" of Whiteley (1954), the "significant fat embolism" of Denman and Gragg (1948), and the "+ fat embolism" of Robb-Smith (1941). The line between moderate and severe fat embolism has been drawn quite arbitrarily at the level above which systemic fat embolism may be expected to occur.

Distribution of fat emboli in the lungs was not studied, but other workers failed to demonstrate any localization (Olbrycht, 1922). No method of selecting blocks for [illegible] owed, save that, where macroscopic appearanecs of various areas of the lungs varied, an endeavour was made to obtain blocks representative of all areas. Each case in both series was examined for pulmonary fat embolism. In these preparations fat emboli were seen as orange-red staining masses in the capillaries and small arterioles, and similar masses often lay free in the alveoli. Fat-containing macropnages were constantly present, but their presence and numbers bore no relationship to the degree of fat embolism (Scott, Kemp, and Robb-Smith, 1942), and they were easily identified. Fat deposits around the larger bronchi and pulmonary vessels were encountered, but the fat could be seen to be extravascular.

The changes in the lung parenchyma accompanying fat embolism were found to be inconstant and non-specific. Transudation of fluid into the alveoli, with infection, diapedesis of polymorphs, and extravasation of red cells are all found in lungs showing no or minimal fat embolism as well as in those with severe degrees of the condition. Conversely, there may be little or no reaction to severe degrees of fat embolism.

Systemic Fat Embolism

Fat emboli originating in the marrow of injured bones traverse the venous system and the right side of the heart and impact in the pulmonary vessels. In the absence of a functioning communication between the pulmonary and systemic circuits emboli appearing in the latter must have traversed the pulmonary capillaries. Possible by-pass routes are:

(1) A patent foramen ovale, which Friedberg (1950) states is present in 20 to 25% of adult hearts but of functional significance in only 6%. In the remainder the opening is covered by a flap valve which is kept closed by the difference in pressure between the atria. Should congestion of the right heart reverse the pressure difference, right to left passage of emboli could occur, and this has been stated to happen (Green and Stoner, 1950).

(2) The bronchopulmonary venous shunt (Marchand, Gilroy, and Wilson, 1950), which is also said to become important if pressure in the right heart rises.

Both these mechanisms depend on a rise in pulmonary arterial and systemic venous pressure. This has been stated to occur in pulmonary fat embolism, but in the present series there has been no clinical evidence to confirm this observation. There is also no evidence that systemic fat embolism is commoner in patients with a patent foramen ovale. It is concluded that when fat emboli gain access to the systemic circulation they commonly do so by passing through the pulmonary capillaries.

The degree of pulmonary fat embolism is one factor determining the occurrence of systemic fat embolism, but other factors remain largely conjectural. Anything changing the diameter of the pulmonary capillaries will affect the passage of emboli; such factors have been described (Swank and Hain, 1952; Swank and Dugger, 1954) and have been suggested to be associated with the production of systemic fat embolism. Systemic embolism has also been stated to be commoner in young people with sound hearts beating forcefully than in the aged (Hämig, 1900). That other factors are involved is seen by the variable occurrence of systemic fat embolism in patients with the same degree of pulmonary fat embolism.

Once fat emboli are released into the systemic circulation their distribution is a random matter, depending on the amount of blood each organ receives. The symptoms produced will depend on the vulnerability of the organ to minor vascular occlusions. Most organs have considerable functional reserve and can withstand blockage of a small proportion of their capillaries without disturbance. The brain is the chief exception to this generalization. The vessels of the cerebral white matter in particular are end-arteries and have poor collateral circulation; cerebral tissues are acutely sensitive to anoxia and vascular lesions are promptly followed by disturbances of function. Anatomical lesions follow and are most conspicuous in the white matter, giving the histological picture of an area of haemorrhage surrounded by one of avascular necrosis and forming the "ball" and "ring" haemorrhages and "anaemic infarcts" of cerebral tissue. Swank and Hain (1952) describe the experimental production of such lesions by the injection of emboli of known size, and their studies emphasized the production of micro-infarcts and the abnormal permeability of capillaries in the absence of other visible abnormality. They saw persistent lesions in the brain of an animal 100 days after a single injection of emboli, and emphasized the speed of functional recovery in the presence of anatomical lesions.

The classical description of gross cerebral fat embolism is of a brain under moderate tension, showing on section multiple petechial haemorrhages throughout the white matter of the cerebral hemispheres, and to a lesser degree in the grey matter of the cortex and in the brain-stem and cerebellum. Lesser degrees of fat embolism, while sufficient to cause gross impairment of function, are not always apparent macroscopically and may be diagnosed on histological evidence only. When cerebral fat emboli are present they are seen lying in the cerebral capillaries, in the centre of small areas of haemorrhage, or in association with anaemic infarcts. In numbers they are scanty compared with pulmonary fat emboli, even in patients whose death is attributable to the cerebral fat embolism. No attempt has been made to count cerebral fat emboli, but their numbers were assessed and their importance in causing symptoms and death determined in relation to the other lesions and the clinical history. Any cerebral fat embolism is of grave significance in contrast to pulmonary fat embolism, which is

an almost constant accompaniment of fractures and of only minor importance.

Non-embolic fat is often found in the brain and has been a source of confusion. Such fat is common in the vessel walls and perivascular tissues of the larger vessels, but no evidence has been found that it is of embolic origin. Swank and Dugger (1954) counted all stainable fat seen in cerebral sections and described it as embolic, but this does not appear to be justified in human material, and only fat seen to be intravascular or in association with infarcts has been accepted as embolic.

It is probable that the effects of cerebral fat embolism are attributable to mechanical occlusion of vessels and tissue anoxia. The possibility of fat having an irritant chemical action has been raised, but histological examination does not suggest that this is significant. Cammermeyer (1953) has described juxta-embolic thrombosis of fibrin complicating fat embolism, and suggests that this is due to anoxic tissue necrosis releasing a thromboplastic substance which diffuses into the vessels. The direct effect of fat on clotting (Fullerton, Davie, and Anastasopoulos, 1953) must also be considered in this connexion, and the effect of increased fat on blood viscosity has been described by Cullen and Swank (1954). They feel that this in itself may be sufficient to cause tissue changes in the absence of actual cerebral embolism.

Sections of kidney were examined for fat emboli in all but one of the trauma series. Systemic fat embolism is generally better seen here than in any other tissue because of the large blood supply and the localization of the capillary bed in the glomeruli. If systemic fat embolism is present it should best be seen in renal sections, and if the emboli are few in number they may be seen nowhere else. Of 24 cases in the trauma series showing systemic fat embolism, only four did not have renal embolism. Renal fat embolism does not produce changes in the renal parenchyma or in renal function. Early attempts were made to implicate renal fat embolism in the production of post-traumatic oliguria and uraemia (Gauss, 1924), but investigation did not sustain this. Flick and Traum (1930) investigated the effects of fat emboli on the kidneys of healthy experimental animals, and showed that only slight changes were produced which resulted in no permanent impairment of renal function.

All sections of brain and kidney examined for fat embolism were cut at 15 μ on the freezing microtome and stained with oil red O and light green.

Systemic fat embolism has been reported to occur in almost every organ, but the only other site in which it has been thought to be of danger to life is the heart. Early descriptions of systemic fat embolism distinguished a coronary type and a fatal syndrome arising therefrom (Gauss, 1924). Sections of myocardium from several patients with severe systemic fat embolism have been examined, but in only one case were emboli found and they did not appear to be of significance. Scully (1956) was unable to identify a syndrome due to myocardial fat embolism.

Results

Control Series.—This consists of 53 patients dying after burns or non-traumatic illnesses. They are analysed in Table I.

TABLE I
CONTROL SERIES

	Age in Decades								Total	Burns	Non-burns
	1	2	3	4	5	6	7	8+			
All cases ...	21	5	4	3	3	4	5	8	53	43	10
Cases with pulmonary fat embolism	5	2	4	1	1	3	2	3	21 (40%)	16 (37%)	5 (50%

The incidence of pulmonary fat embolism is high, occurring in 37% of the burns cases and in 50% of the other cases. However, of 16 burns cases showing pulmonary fat embolism, 13 showed a count of 5 or less, and these minimal numbers are not considered significant. The remaining three cases had counts of 13, 16, and 21. Of the 16 cases none showed symptoms of pulmonary or systemic fat embolism, and in those where renal and cerebral sections were examined for fat emboli none were found.

In the "non-burns" series five cases showed pulmonary fat embolism. In three the count was less than 5, and in one it was 6. In the remaining case a man dying of a ruptured cerebral aneurysm had pulmonary fat embolism with a count of 47, but no systemic emboli. The onset of the haemorrhage had caused him to fall 4 ft. from a ladder, and he suffered lacerations but no fractures. The moderate pulmonary fat embolism probably resulted from general skeletal shaking.

Trauma Series.—This consisted of 100 patients dying after injury. In most cases the injury was the immediate or underlying cause of death. The group is analysed in Table II; the preponderance of males reflects the greater liability of men to severe trauma in all but the highest age groups.

TABLE II
TRAUMA SERIES

	Age in Decades								Total
	1	2	3	4	5	6	7	8+	
Males	2	4	12	6	11	12	11	13	71
Females	1				3	3	6	16	29
Both sexes	3	4	12	6	14	15	17	29	100

Only in the over-71 age group is the number of women greater than that of men; of these old ladies, 10 died after a fracture of the neck of the femur.

Classification of Trauma.—Trauma is divided into three grades :

" Mild " denotes fractures of the skull, or a fracture of the neck of the femur.

" Moderate " denotes fracture of one long bone, or two or three fractures of any bones.

" Severe " denotes four or more fractures.

The generally accepted source of fat emboli is the marrow of fractured bones, and in considering the relationship between fat embolism and trauma the number of fractures is of prime importance. Only very severe soft tissue injuries were taken into account in a few cases in assessing the degree of trauma.

Eighty-nine of the 100 cases showed pulmonary fat embolism. In the 11 cases where none was found, two factors were considered : (1) In all the survival time was long, averaging 40 days after injury and in no case being less than 12 days. (2) In several of the 11 cases the degree of trauma was very slight. In the remaining 89 cases it has been found that in general the degree of pulmonary fat embolism increases with the severity of trauma, severe fat embolism being uncommon with mild and most common with severe trauma (Table III).

TABLE III
RELATIONSHIP BETWEEN DEGREE OF TRAUMA AND OF PULMONARY FAT EMBOLISM

		Degree of Trauma			Total
		Mild	Moderate	Severe	
Pulmonary fat embolism	Mild ..	17	6	5	28
	Moderate	7	9	13	29
	Severe ..	3	4	25	32
	Total	27	19	43	89

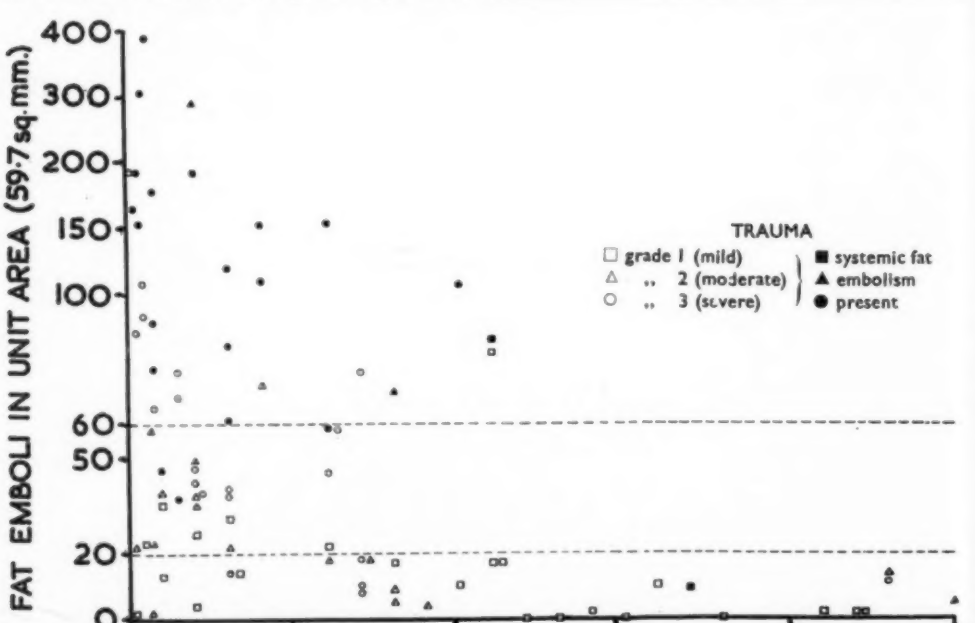

FIG. 1.—Incidence and severity of fat emboli after trauma related to time after injury.

Conversely, mild fat embolism is usually associated with mild trauma, but the correlation is not so close. The longer a patient survived after injury, the less the degree of fat embolism found (Fig. 1).

Incidence of Systemic Fat Embolism.—Each case of the trauma series was investigated for the presence of systemic fat embolism. Whenever possible sections of both kidneys and brain were examined, but both tissues were not available in every case. In no case where pulmonary fat embolism was absent was systemic fat found. Ninety-nine of the 100 cases were examined for renal fat emboli. The one case that was omitted showed neither pulmonary nor cerebral emboli. In 30 cases no sections of brain were available; in three of these renal fat emboli were present, but in the absence of cerebral sections the existence and significance of cerebral fat embolism had to be inferred from the degree of renal fat embolism and the clinical history.

Twenty-four of 100 cases showed systemic fat embolism. Distribution in respect of degree of trauma and of pulmonary fat embolism is analysed in Table IV. Of the 24 cases, 19 (80%) were

TABLE IV

CASES WITH SYSTEMIC FAT EMBOLISM CLASSIFIED BY DEGREE OF TRAUMA AND OF PULMONARY FAT EMBOLISM

		Degree of Trauma			Total	Total as Percentage of Cases in Group
		Mild	Moderate	Severe		
Pulmonary fat embolism	Mild ..	1			1	3·5
	Moderate			4	4	14
	Severe ..	1	2	16	19	61
Total ..		2	2	20	24	24
Total as percentage of cases in group		7	10	45	24	

patients with severe pulmonary fat embolism, 20 (83%) had suffered severe trauma, and 16 (66%) fell into both groups. This demonstrates the close relationship between severe trauma, severe pulmonary fat embolism, and systemic fat embolism. There remains one case in which mild trauma and mild pulmonary fat embolism are associated with systemic fat embolism; this has not been explained. Apart from this case, conclusions from this series confirm those of Scully (1956).

Effects of Pulmonary Fat Embolism.—Opinions differ widely on the significance of pulmonary fat embolism, ranging from the belief that it is the cause of grave symptoms and death in a high proportion of accident cases (Warthin, 1913; Robb-Smith, 1941; Denman and Gragg, 1948; Glas, Grekin, and Musselman, 1953) to the opinion that it is only rarely the cause of symptoms (Wilson and Salisbury, 1944; Scully, 1956). Results of the present series reinforce the latter opinion, and have failed to confirm the existence of a syndrome due to severe pulmonary fat embolism. Characteristic gross findings were not apparent at necropsy, and study of the clinical records for unexplained pulmonary symptoms, e.g., cough, dyspnoea, bronchitis, pneumonia, collapse, or pulmonary oedema, showed that these were at least as common in cases with mild pulmonary fat embolism as in those where the embolism was severe. Of 35 cases of mild pulmonary fat embolism, nine (26%) showed such symptoms before death; of 28 cases of severe pulmonary fat embolism, four (14%) showed such symptoms. It is possible that pulmonary fat embolism might so affect a lung with a reduced reserve from pre-existing disease as to produce acute pulmonary insufficiency, and symptoms thought to be due to this were seen in one case in this investigation.

Effects of Systemic Fat Embolism.—While pulmonary fat embolism is a regular and relatively unimportant finding after fractures, systemic fat embolism was only found in 24% of the trauma series. Four cases showed cerebral fat emboli but no renal fat emboli. In two of these the absence of renal fat emboli is attributed to renal ischaemia in post-traumatic shock, as both were known to have been hypotensive for some hours and in one the kidneys showed ischaemic tubular necrosis. In the other two cases no explanation for the sparing of the kidneys could be found. A complete study of the necropsy findings and clinical records was made in 23 of the 24 patients; in the omitted case the clinical notes were not available.

The cases were separated into three groups: (1) Systemic fat embolism not contributory to symptoms or death (11 cases); (2) systemic fat embolism of doubtful significance in relation to symptoms and death (six cases); (3) systemic fat embolism giving rise to symptoms and death (seven cases). Of these seven cases (*a*) in four the systemic fat embolism was a contributory cause of death, and (*b*) in three systemic fat embolism was the main cause of death.

Comment

The frequency of fat embolism will always be found to be higher when assessed on histological as opposed to other criteria, as mild degrees of embolism may not give rise to symptoms. In previously published series the incidence of pul-

monary fat embolism found by histological examination of material from general necropsy series or in cases where injury was not the cause of death has ranged from 2% (Warthin, 1913) to 52% (Wright, 1932). The proportion considered to be of significance in causing symptoms was estimated at 1 to 2% (Warthin, 1913 ; Denman and Gragg, 1948). The proportion of cases of death after injury showing pulmonary fat embolism has been estimated at from 50% (Denman and Gragg, 1948) to 100% (Wyatt and Khoo, 1950). The number of deaths due to pulmonary fat embolism was estimated as 2% (Vance, 1931), 30% of 70 deaths after fracture (Robb-Smith, 1941), and 37% of 19 deaths from injury (Denman and Gragg, 1948).

When systemic fat embolism is assessed separately only Scully (1956) gives figures based on histological criteria. He found systemic fat embolism in 17% of fatal battle casualties, and considered death to be due to the condition in 1%.

Wilson and Salisbury (1944), using clinical criteria, found an incidence of systemic fat embolism of 0.8% in 1,000 battle casualties with death in 0.6%. Newman (1948), using clinical criteria, found an incidence of 6% of systemic fat embolism, with death in 3%, in a series of 89 injuries to long bones. Glas *et al.* (1953) estimated the incidence of systemic fat embolism in 109 cases of injury as 14% and thought death was due to it in 5.5%.

Not all these results are strictly comparable, as criteria applied vary between them. It is hoped that the strict quantitative histological criteria for the estimation of fat embolism, combined with study of clinical records, may serve as a reference in future investigations of the problem.

Removal of Fat Emboli.—Fat emboli are gradually removed from the tissues where they impact ; Fig. 1 shows the steady decline in numbers of pulmonary fat emboli as the survival period after injury increases. Experimentally produced fat emboli are seen in the lungs " within seconds of injury " (Glas *et al.*, 1956). Maximal numbers are found in patients dying within 48 hours of injury, and after this there is a fairly steady decline. The time taken for the emboli to traverse the pulmonary capillaries and impact in the systemic vessels is less certain. The rule generally quoted for the signs of cerebral fat embolism is " fat embolism, 3 days," but the time lapse after injury is now generally thought to be shorter than this (Alldred, 1953) and in three patients in the present series the signs of cerebral fat embolism were apparent within 24 hours of injury.

Loss of fat emboli has been suggested to take place in the sputum, in the urine, by phagocytosis and by lipases. Large numbers of free fat globules can be found in the sputum after injury, and while this is not pathognomonic of fat embolism (Nuessle, 1951) it does show that a considerable amount of fat can be lost in this way. Fat does appear in the urine after injury, but it appears late and is difficult to detect, which suggests that quantitatively this route of elimination is not important.

Disappearance of fat emboli from the lungs can be demonstrated in those cases where an infarct preserves the number of emboli at the time of infarction, while in the remainder of the lung removal proceeds normally. In one such case there was survival for 23 days after injury. A lung infarct found at necropsy could be correlated with an episode of chest pain and haemoptysis seven days after injury. The count of emboli in the uninfarcted lung was 13 ; in the infarct it was 114, showing that in 16 days 101 emboli per unit area, or 88% of those present, had been removed.

Summary and Conclusions

Clinical records, necropsy findings, and histological preparations stained for fat have been studied in 100 patients who died after suffering injury and in 53 patients who died from burns or causes not due to injury. Pulmonary fat embolism was assessed quantitatively by counting emboli in a unit area of standard section and was classified as mild, moderate, or severe. In all patients of the trauma series and in some of the control series an examination was made for systemic fat embolism in kidney, brain, or both tissues.

Forty per cent. of the control cases showed pulmonary fat embolism, but in all save a few it was minimal and insignificant. In no case of the control series was systemic fat embolism found, nor were there symptoms suspicious of systemic embolism.

Eighty-nine per cent. of patients dying after injury showed pulmonary fat embolism. In the 11% without pulmonary fat embolism the average period of survival after injury was 40 days and the shortest 12 days. In no case was systemic fat embolism found in the absence of pulmonary fat embolism.

Systemic fat embolism was found in 24% of the patients dying after injury. In 11% it was considered to be of no significance, in 6% of doubtful significance, in 4% it was a contributory cause of death, and in 3% the chief cause of death.

Pulmonary fat embolism increases in severity with the degree of trauma. Systemic fat embolism is commonly found only in patients with severe pulmonary fat embolism.

The existence of a syndrome, or of characteristic gross necropsy appearances, due to severe pulmonary fat embolism has not been confirmed. Pulmonary fat embolism is not thought to be of importance as a cause of illness or death. Cerebral fat embolism is more important as a cause of symptoms and death than is commonly realized, but does not always give rise to the syndrome described as characteristic.

I am indebted to Dr. S. Sevitt for access to the pathological records and material, and for advice and encouragement throughout the work. My thanks are due to the surgeons of the Birmingham Accident Hospital for permission to utilize their clinical records, to Mrs. J. Priest and Mr. A. Randle for histological preparations, and to Mr. H. Lilly for Fig. 1.

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TRAUMATIC DISSECTING ANEURYSMS

BY

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Most dissecting aneurysms of the aorta are spontaneous, and have as their underlying pathology a degeneration of the media. Strain, physical and mental, has been suggested as a factor which precipitates their onset, but external trauma is usually exonerated. Shennan (1934) traced only six cases due to trauma, and he formed the opinion that there was rarely a clear relationship between these aneurysms and external trauma. When Mote and Carr (1942) reviewed 60 cases of dissecting aneurysm investigated in the San Francisco Coroner's Office, they concluded that external trauma was not a precipitating factor. Subsequent reviews, such as those of Gore (1952), Gore and Seiwert (1952), and Graham and Milne (1952), omit any mention of this factor.

This belief that dissecting aneurysms arise independently of external violence is wrong. Some dissecting aneurysms are precipitated by trauma and they have features which appear to differentiate them from the spontaneous kind. An awareness of these features will show that these aneurysms are not as rare as was previously supposed.

The following three cases occurred in Leeds during the last two and a half years.

Case Reports

Case 1.—A motor bus driver, aged 66, having reached the terminus, jumped out of his cab, a distance of 3 ft. from the ground, and landed heavily on his feet. As he did so he felt an excruciating pain in the lower part of his back, and, after staggering to the rear of the bus, he was helped to a seat inside. When an ambulance arrived he was in great pain and semiconscious. He was taken to hospital, where he died the following day.

At necropsy, by Dr. William Goldie, 3–4 litres of fluid blood and blood clot were found in the right pleural cavity and the right lung was completely collapsed. There was a transverse tear, less than 1 cm. long, of the aortic intima, at the junction of the arch with the descending aorta, and dissection of the wall extended from this point distally for 6 in. to where the aneurysm had ruptured into the right pleural cavity. In this situation there was a second intimal tear through which the aneurysm again communicated with the lumen of the aorta. The artery was moderately atheromatous (St. James's Hospital, Leeds, P.M. No. A 580).

Case 2.—A man, 60, was a passenger in a motor car which ran into the back of a stationary vehicle and was itself run into by a car from behind. The passengers were shaken considerably and the precise course of events was obscured by the confusion that followed. Within half an hour of the accident, however, the man complained of pains in his back as a result of the collision. It was over two hours before he arrived home, and he then found himself unable to lie in bed because of the pains in his chest. As a consequence he spent the night in a chair before the fire. He was seen by the doctor the next morning and he stayed in the chair for a further 24 hours, when he was carried upstairs to bed. The following day he became very ill and he died in an ambulance while on his way to hospital 61 hours after the accident.

At necropsy, 3 pints of blood were found in the left pleural cavity. There was a horizontal tear, 2 cm. long, of the intima of the left wall of the aorta at the junction of the arch and the descending portion. Dissection had extended upwards over the arch and downwards as far as the origin of the left renal artery. Death occurred when the aneurysm burst into the left side of the chest. The aorta was moderately atheromatous. The heart weighed over 500 g. due to hypertrophy of the left ventricle, the wall of which was 20 mm. thick. This was consistent with hypertension (Department of Forensic Medicine, University of Leeds, P.M. No. 4501).

Case 3.—A woman aged 75 years was crossing the road at night when she was struck by a car travelling at about 40 m.p.h. After the impact she was carried about 12 yards by the vehicle. She was dead when seen by a doctor shortly afterwards.

At necropsy, there was a fracture of the cervical spine with severance of the cord at the level of the third cervical vertebra, extensive bruising of the tissues of the neck and mediastinum, bilateral haemothorax, subarachnoid haemorrhage, and multiple fractures. There was a transverse tear, 4 cm. long, of the intima of the posterior wall of the thoracic aorta at the junction of the arch and the descending portion. A

small dissecting aneurysm which admitted the tip of a finger had formed. Its outer coat had ruptured and blood had entered the mediastinum (Department of Forensic Medicine, University of Leeds, P.M. No. 3508).

There is no doubt that these three dissecting aneurysms were precipitated by external violence. In the first two cases the relationship of the trauma to the aneurysm is confirmed by the onset of pain in the chest immediately after the injury. Pain radiating from the mid-thorax is a common clinical feature of dissecting aneurysm ; it may be severe, as in the first case, or mild, as in the second. In the third case it is impossible accurately to determine the onset of the aneurysm, but the necropsy findings, together with the evidence given in the coroner's court, leave no doubt that it was the result and not the cause of the accident.

Traumatic dissecting aneurysms follow tearing of the inner layers of the aortic wall, a change which can be regarded as an incomplete aortic rupture.

In spite of the apparently protected position of the thoracic aorta, rupture of its wall by trauma is not as rare as we are led to believe. Copeland (1914), Wilson and Roome (1933), Collins and D'Alessio (1938), Kleinsasser (1943), and McDonald and Campbell (1945) remark on the rarity of traumatic aortic rupture. In the necropsy practice of the Department of Forensic Medicine, University of Leeds, however, rupture of the aorta is often found. Of 85 persons who died by trauma during the five-year period of 1951–56, the aorta was ruptured in no less than 24 (27.2%). The rupture was situated in 19 (79%) at the junction of the arch and the descending part, which thus shows that this segment is the most vulnerable part of the artery.

Small intimal tears, ¼–½ in. in length, which could have been starting points of dissecting aneurysms, were also seen. In each of two persons, one a man of 40 years (P.M. 2634) buried by a fall of earth and the other a man of 23 years (P.M. 4521) who was a passenger in a car which struck a lamp standard, the aorta was torn across at the junction of the arch and the descending part and small circumferential tears of the intima were found in the adjacent distal segment. Another man had fallen down a lift shaft, and, although there was no rupture of the aorta, several dozen circumferential tears, up to ¼ in. in length, were found in the intima (P.M. 3445). Tears of this nature may heal ; intimal scars believed to be healed tears have been described by Lundberg (1930) and by Peery (1942).

The commonest injury of the aorta, then, is a complete tear of its wall over an arc or the whole circumference, resulting in death from haemothorax. In some cases, however, the intima alone is torn ; this may heal or may become the starting point of a dissecting aneurysm. Death may occur, as in the third case, before dissection is appreciable or, as in the first two cases, the person may live long enough for dissection to be extensive. In common with spontaneous dissecting aneurysms it is presumably possible for traumatic aneurysms to heal. Samson (1931) described two cases of dissecting aneurysm which he ascribed respectively to trauma five years and 11 years previously.

The site of the intimal tear is important. The commonest site of aortic rupture is at the junction of the arch and the descending part. In each of the present dissecting aneurysms the intimal tear was also in this region. This indicated a traumatic origin of these aneurysms, for, contrary to general belief, the intimal tears associated with spontaneous dissecting aneurysms are infrequent at this junction. A survey of the departmental records covering the five-year period 1951–56 showed that there were 47 spontaneous dissecting aneurysms. Intimal tears in 29 (62%) of these cases were in the ascending aorta and in 13 (28%) they were in the arch. In only two cases was the tear at or near the junction of the arch and the descending aorta, and these cases had special features. In one (P.M. 3840) the dissection was slight and related to the immediate vicinity of an irregular tear in the wall of an atheromatous aorta which was extensively calcified and " ulcerated." The other case (P.M. 4112) was one of a " double aorta " where dissection had extended into the abdominal portion as far as its bifurcation.

The susceptibility of this part of the aorta to trauma depends on the anatomy of the region. The segment of the artery most commonly ruptured lies between the attachment of the ligamentum arteriosum and the origin of the first aortic intercostal arteries. The part of the artery below this segment is anchored to the spine by the pleura and the intercostal arteries. The part above it is firmly held between the great vessels of the neck and the structures within the arch, and moves with these structures whenever the mediastinum is displaced. In so doing a strain is imposed on the segment of the aorta which lies between the ligamentum and the first aortic intercostal arteries. Anything which causes violent displacement of the mediastinal structures, such as a sudden blow on

the chest or rapid deceleration of the trunk, is likely to tear the aorta in this region.

It may be that a tear occurs more readily during systole when the aorta is tense. Two other factors may also predispose to tearing. Sometimes a calcified plaque up to 2 cm. in diameter forms in the aortic wall at the point of attachment of the ligamentum arteriosum. When this is present, the strain on the aortic wall when the ligamentum is tensed is doubtless increased.

The other factor is the sudden and violent compression of the aorta against the thoracic spine by the parietal pleura which is tensed whenever the hilum of the left lung is displaced forwards. Under experimental conditions, tensing of the pleura extending from the left hilum to the ribs can be shown to kink the aorta at a point corresponding to the usual site of rupture.

Recognition that dissecting aneurysms can be either spontaneous or traumatic has medico-legal importance. Their accurate distinction enables civil liabilities to be apportioned and helps in the settlement of claims under accident policies. The interpretation that the aneurysm in Case 2 was traumatic enabled the widow to claim successfully against the insurers of the motorist.

Whenever the intimal tear of a dissecting aneurysm of the aorta is found at about the junction of the arch and the descending aorta, trauma is the probable cause.

Summary

Three cases of traumatic dissecting aneurysm are reported. Features are described by which dissecting aneurysms of traumatic origin may be distinguished from those arising spontaneously.

I am indebted to Professor C. J. Polson for his help in the preparation of this paper. I wish to thank Dr. William Goldie for allowing me to make use of his post-mortem records of Case 1, and to Mr. R. E. Nutt, H.M. Coroner for the City of Leeds, for permission to publish these cases.

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ANTITHROMBOPLASTIN: THE DEGENERATION OF INTRINSIC THROMBOPLASTIN IN NORMAL SERUM

BY

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It is well known that a powerful thromboplastin is generated during blood coagulation, and a high titre of thromboplastin can be demonstrated in specimens of fresh serum. Its generation may be observed by means of the thromboplastin generation test (Biggs and Douglas, 1953) when a mixture of antihaemophilic globulin, Christmas factor, platelets, factor V, and factor VII is recalcified. If this test is prolonged beyond the usual six-minute incubation period, it will be noticed that the thromboplastin titre rapidly falls; a mixture, which after five minutes' incubation coagulates the substrate plasma in 10 seconds, may take well over 40 seconds to clot the same plasma after another 10 minutes' incubation. Although this degeneration of thromboplastin must have been noticed by all who have performed the test, there appears to have been little research into the mechanism of its removal. There would seem to be two possibilities: either thromboplastin deteriorates by virtue of its own physical instability, or it is destroyed by a specific antithromboplastin which may be present in plasma or serum.

Tocantins has postulated the existence of an antithromboplastin, by which he means an inhibitor of thromboplastin formation (1954). Similarly, circulating anticoagulants, described by Lawrence and Johnson (1942), Munro (1946), Fantl and Nance (1946), Biggs and Macfarlane (1953), Hardisty (1954), Hougie and Fearnley (1954), and many other workers, have no action on formed thromboplastin, and are more correctly described as antithromboplastinogens.

During the course of work at this laboratory, the ability of serum to destroy brain thromboplastin was observed, and it has been postulated that there is present in serum an antithromboplastin, the function of which is to remove tissue products and extrinsic thromboplastin from the circulating blood (Berry, 1957). Following these experiments a simple test was devised to follow the rate of degeneration of naturally-produced intrinsic thromboplastin, liberated during coagulation in specimens of fresh serum. This test was used in an endeavour to confirm the existence of an antithromboplastin in normal serum. The technique of the test and the results obtained are described in the present paper.

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Reagents

The specimens of blood examined were obtained by venipuncture from members of the laboratory staff, and from surgical patients shortly after their admission to hospital and at intervals after operation. Blood obtained after a difficult venipuncture (whenever there is excessive tissue trauma, or whenever there is possible platelet destruction during a slow collection) is unsuitable for the test.

Physiological calcium-fibrinogen-saline was prepared for use as a diluent. This consisted of normal saline containing 10 mg. of calcium chloride and 200 mg. of fibrinogen per 100 ml.

Fibrinogen was supplied by the Lister Institute. Each ampoule was made up with 10 ml. of distilled water, and subsequently diluted to give a concentration of 200 mg. %.

To avoid further dilution of serum specimens, a set of glass tubes was prepared, each of which contained the dehydrated equivalent of 0.2 ml. of M/40 calcium chloride.

Technique of the Test

Blood, 15 ml., is collected, 10 ml. of which is citrated (0.6 ml. citrate/5 ml. blood) and received into siliconed glass tubes. The remaining 5 ml. is left uncitrated in another siliconed tube. All specimens are immediately placed in a beaker of melting ice which is taken to the patient's bedside.

As soon as possible after venipuncture all specimens are centrifuged at 1,000 r.p.m. for five minutes. The uncitrated specimen of plasma is then transferred (using a siliconed pipette) to another siliconed tube and returned to the beaker of melting ice. It will remain unclotted long enough for the completion of the other preparations for the test.

The platelet-rich supernatant plasma from the citrated blood is centrifuged for a further 15 minutes at 3,000 r.p.m. The resulting platelet-free supernatant is used as the substrate plasma during the test. This specimen is returned to the beaker of melting ice, and it will remain at a temperature of 0° C. during all subsequent procedures.

In a rack in the 37° water-bath are placed a number of tubes containing dehydrated calcium chloride.

When these preparations have been completed, 20%, 10%, and 1% dilutions of the uncitrated specimen of plasma are prepared in non-siliconed tubes, using the calcium-fibrinogen-saline as the diluent. The remaining undiluted plasma and the three dilutions are then transferred to the water-bath, each one being thoroughly agitated using a wooden applicator stick, where they will coagulate. The clotting times are observed, and also the time when each one clotted. Ten minutes after coagulation the fibrin is removed from each tube, the contained serum being expressed. The more dilute specimens may show a continued deposition of fibrin, and this is removed as it forms.

At intervals of 15, 30, 45, 60, 90, 120, and 180 minutes after coagulation, specimens from each dilution are examined for ability to promote coagulation of the substrate plasma as follows: 0.2 ml. of the serum is transferred to one of the tubes containing calcium chloride, and the tube is agitated to ensure complete solution of the calcium. Then 0.2 ml. of substrate plasma is added to the contents of this tube, and the clotting time of the mixture is noted. The serum is added to the calcium chloride immediately before each clotting time is to be performed.

In order to determine the effect of residual thrombin which may be present in the serum specimens, a parallel series of clotting times was performed, the procedure being the same as that described above, but using 0.2 ml. of fibrinogen solution in place of the substrate plasma.

By using saline instead of serum, the calcium clotting time of the substrate plasma was determined in every case.

Results

The clotting times of the uncitrated plasma, undiluted, after transferring from melting ice to the water-bath, varied from 200 to 420 seconds in 38 cases investigated. Dilution of the plasma with calcium fibrinogen saline did not result in a very great increase in the clotting time; the difference between clotting times of the 100% and 1% specimens was never greater than five minutes. Omission of calcium chloride from the mixture leads to considerable prolongation, however, some specimens taking as long as one hour before deposition of fibrin was observed.

The calcium clotting times of substrate plasma from these 38 cases varied from 150 to 344 seconds.

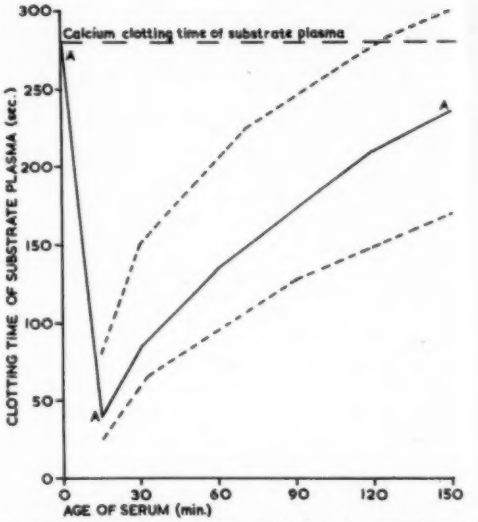

FIG. 1.—Graph showing the ability of different aged specimens of serum to bring about the coagulation of substrate plasma. The graph A–A–A represents the average readings, the full range of which lies between the two outer curves.

The clotting times of the substrate plasma when added to 15-minute-old serum varied from 25 to 62 seconds, the average time being 40 seconds. With increasing age of the serum, the plasma clotting time also increased, until eventually the calcium clotting time figure was reached. Specimens of serum over two hours old were able to bring about little or no shortening of the substrate plasma clotting time. These results are shown in Fig. 1.

Dilution of the uncitrated specimens before coagulation did not greatly affect the ability of fresh (15 minute) serum to reduce the clotting time of the substrate plasma. The difference between the "clotting activity" of undiluted specimens and the 1% dilutions, represented in terms of clotting time, was never more than 50 seconds. Whereas, however, the undiluted serum rapidly lost its ability to shorten the clotting time of substrate plasma, this deterioration was far slower in the 20% dilution. The 10% and 1% specimens showed little loss of potency even after several hours' incubation (Fig. 2).

Repeating the investigations, with fibrinogen solution in place of the substrate plasma, gave an indication of the part played by thrombin in producing these results. It was observed that, even though fibrinogen clotting times were always greater than the corresponding substrate plasma

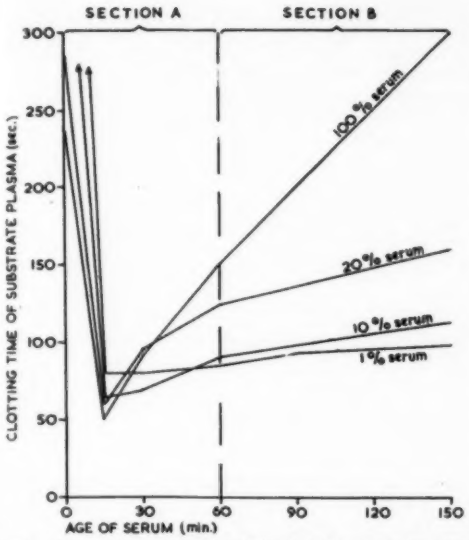

FIG. 2.—Graph showing the effect of dilution of serum on its coagulant activity, and on the subsequent deterioration of this activity.

clotting times, serum specimens still contained a significant amount of thrombin up to 45 minutes after coagulation. Older specimens of serum contained little if any thrombin, however, and the fibrinogen clotting times of serum specimens older than 45 minutes were almost invariably greater than 10 minutes.

Interpretation of Results and Discussion

A fresh specimen of serum may contain unconverted prothrombin, thrombin, and factor VII, in addition to the thromboplastin generated during coagulation. Each of these may be concerned in the coagulation of the substrate plasma. In the absence of platelets in the substrate, it is believed that Christmas factor in the serum will not be involved, and can be ignored when interpreting the results of the test.

In a normal specimen, having a normal rate of prothrombin conversion, it is believed that the amount of prothrombin remaining in a 15-minute-old specimen of serum will be insignificant compared with the amount in the substrate plasma. This residual prothrombin will have little effect in determining the plasma clotting time.

Thrombin is rapidly removed from serum after coagulation, by taking part in the reaction converting fibrinogen to fibrin, by adsorption on to the fibrin clot, and by the action of antithrombin. It has been demonstrated that following the recalcification of a citrated specimen of plasma, most of the thrombin has disappeared from the resulting serum four minutes after clotting (Biggs and Macfarlane, 1953; Berry, 1956). It was assumed for the purpose of the present investigations that thrombin disappeared with equal rapidity after the coagulation of whole blood (or uncitrated plasma). To encourage this, the fibrin clot was allowed to remain undisturbed for 10 minutes before the serum was expressed from it and the clot removed, in order that as much thrombin as possible might be adsorbed.

It was observed, however, when parallel fibrinogen clotting times were performed, that significant amounts of thrombin could be demonstrated in the serum as long as 45 minutes after coagulation. The thrombin persisted for the longest time in the diluted specimens, presumably a result of delayed prothrombin conversion, and of dilution of antithrombin. When interpreting the results, therefore, it was considered that the earlier readings (represented in section A of Fig. 2) were produced by the combined effect of thrombin and thromboplastin, and only those readings from older serum specimens (section B, Fig. 2) could be expected to reflect thromboplastin activity alone.

In the absence of platelets there can be no liberation of intrinsic thromboplastin in the substrate plasma itself. It is thought that even the most carefully collected specimen of blood must contain traces of tissue fluid; for this reason specimens were collected and stored in melting ice to discourage the production of extrinsic thromboplastin in the substrate plasma.

The results of these experiments have shown that fresh serum (up to three hours after coagulation) has coagulant properties, and that with increasing age the serum loses this ability to clot the substrate plasma. The shortening of the plasma clotting times is greater than can be explained by the presence of prothrombin or thrombin in the serum, and must depend largely on the thromboplastin content of the serum. A study of the results as shown in Fig. 2, and especially those results in section B of the graph, suggests that this thromboplastin rapidly degenerates, and that the rate of degeneration varies with the dilution of the serum, being most rapid in the undiluted specimens. In fact the most dilute specimens showed little degeneration over a period of three hours.

If thromboplastin is a labile substance, and disappears from serum because of its lability, one would expect the rate of degeneration to be constant in all dilutions of serum. The varying rates

of degeneration shown by these experiments are in favour of the existence of an antithromboplastin in normal serum.

Summary

Investigations into the rate of degeneration of intrinsic thromboplastin in fresh serum are described.

It has been shown that the rate of thromboplastin degeneration varies with serum dilution, being more rapid in the more concentrated specimens.

It is suggested that these results can only be explained by postulating the existence of a specific antithromboplastin in normal serum.

I wish to thank the surgical and nursing staff of St. James' Hospital for their co-operation during these investigations, and especially Mr. N. C. Tanner and Mr. A. M. Desmond, under whose care the patients were admitted. I would also like to thank Dr. G. T. Allen for his advice and assistance, and for reading the manuscript.

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PLACENTAL TRANSFER OF THE LUPUS ERYTHEMATOSUS FACTOR

BY

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Records of the placental transfer of the lupus erythematosus factor are scanty. We have been able to find two cases only in the literature (Bridge and Foley, 1954). They described a case where a woman suffering from acute systemic lupus erythematosus was successfully delivered of a normal child. The child remained clinically well, but the characteristic L.E. cells described by Hargraves, Richmond, and Morton (1948) were demonstrated in the blood at birth. The test gave a doubtful result after seven weeks and was negative at four months. They mention one other similar case but give no details.

The production of these cells is now recognized as due to a factor associated with the gamma-globulin fraction of the plasma. An instance is described where this plasma factor was present in the blood of a foetus aged 20 weeks, whose mother suffered from subacute systemic lupus erythematosus. Presumably this was the result of passive transfer of the L.E. factor across the placenta, since at necropsy the foetus showed no evidence of the disease.

Case Report

The patient was a 30-year-old housewife who was quite well until June, 1952, when she was four months' pregnant. She then noticed pain in the knees, ankles, and feet on movement. Subsequently these pains spread to other joints in the body. In September she developed a continuous, dull ache in the left shoulder accompanied by a pleuritic pain in the left lower chest. There was general malaise and excessive sweating at this time. Two weeks before delivery she developed precordial discomfort which was worse on lying flat. At full term, on October 30, she was delivered of a normal female child, weighing 5 lb. 7 oz.

After delivery she ran a continuous fever. On examination on November 15 she had the classical signs of a pericardial effusion which was confirmed radiologically. The B.S.R. was 145 mm. (Westergren) in the first hour and aspiration of the pericardial fluid showed a straw-coloured fluid with a preponderance of lymphocytes. On November 21 she became breathless and was found to have bilateral pleural effusions confirmed radiologically and by aspiration. On December 4 an erythematous rash was noticed on the trunk and all four limbs, and on January 1, 1953, Hess's capillary test was positive. The Mantoux reaction was persistently negative and repeated examinations failed to demonstrate L.E. cells. On May 27, however, one L.E. cell was discovered and one month later moderate numbers were confirmed. On June 1 a course of cortisone was started, being continued with a maintenance dose of 25 mg. three times daily. On this treatment she gained weight, the B.S.R. (Westergren) fell to 24 mm. in the first hour, and her general health improved so that she was able to manage to care for her child and home except for the heavy housework.

In the second pregnancy, which was started against medical advice, she complained of increasing joint pains and her B.S.R. (Westergren) was found to be 112 mm. in one hour. Abdominal hysterotomy was undertaken by Mr. H. G. E. Arthure on January 28, 1956, when the patient was 20 weeks pregnant. Examination of the mother's blood at the time of operation showed approximately one L.E. cell per 500 white cells, and the direct Coombs test was positive in five minutes. The Kahn reaction was positive, but the Wassermann reaction was doubtful, showing a negative reaction with standard antigen and a weekly positive reaction with cardiolopin antigen.

Examination of the blood from the umbilical vein of the foetus showed occasional L.E. cells (less than 1 per 1,000 white cells) and the direct Coombs test was positive in five minutes. Both the Kahn and Wassermann reactions were negative. Filter-paper electrophoresis showed a heavy gamma globulin band in the mother's serum, but this was absent in the amniotic fluid and in the foetal serum.

At necropsy, the foetus appeared normal for the period of gestation with no evidence of the characteristic fibrinoid necrosis in the arterioles or connective tissue of the body including the spleen, kidneys, myocardium, skin, and placenta.

The patient improved clinically, but examination for L.E. cells showed 3 per 200 white cells on February 10 and 10 per 200 white cells on April 25 (three months after operation). On her readmission

to hospital in August, 1956, for investigation of lumbo-sacral pain, she was found to be running an intermittent fever with a B.S.R. (Westergren) of 119 mm. in the first hour. Examination for L.E. cells showed 14 per 500 white cells.

Comment

In this case the investigations show that the test for L.E. cells and the direct Coombs test were positive in both the mother's serum and that of the foetus; the Kahn and Wassermann reactions were positive in the mother only (see Table).

TABLE
BLOOD FINDINGS IN FOETUS AND MOTHER COMPARED

	L.E. Cells	Coombs Test	W.R.	Kahn Reaction	Gamma Globulin*
Foetus	Present	+	−	−	Absent
Mother	,,	+	±	+	Present

* The presence of gamma globulin refers to a visible band on serum electrophoresis.

These results can be explained by passive transfer across the placenta, but the antibodies responsible for these different phenomena appear to cross the placental barrier independently of one another. The transfer of the factor producing a positive direct Coombs test in this condition may obviously cause difficulty in the diagnosis of haemolytic disease of the newborn.

To decide whether to terminate pregnancy in a case of systemic lupus erythematosus is difficult. Many cases start during pregnancy (Guion and Adams, 1943; Graffin, Taylor, and Hass, 1949; Ellis and Bereston, 1952; Smith, 1952; Cohen and Cadman, 1953; Harvey, Shulman, Tumulty, Conley, and Schoenrich, 1954). Moreover, Ellis and Bereston (1952) found that the disease became worse during pregnancy in 28 out of 69 cases. The disease started in our patient during her first pregnancy and became worse during the early weeks of her second. However, some cases improve during pregnancy but become worse in the puerperium (Guy, 1952; Harvey *et al.*, 1954). From a study of the literature only one factor has been discovered which allows a prediction of the effect of pregnancy, or its termination, on the disease; this is the effect of a previous pregnancy. For instance, Rothman (1952) cites a case in which symptoms recurred during the first trimester of three successive pregnancies followed by rapid improvement after abortion, which was either spontaneous or induced therapeutically. This patient, like that reported by Shearn and Pirofsky (1952), improved, but Harvey *et al.* (1954) report cases in which therapeutic abortion led to no improvement.

Although antibodies have been found in the foetus on two previous occasions when systemic lupus erythematosus occurs during pregnancy, no characteristic pathological changes have yet been reported. The high rate of foetal loss (32%) reported by Ellis and Bereston (1952) would thus appear to be due to maternal causes, rather than to a direct effect of the disease on the foetus.

We wish to thank Dr. N. S. Plummer for permission to study this patient and for much helpful advice and criticism, and Dr. John Shore for his necropsy report.

Addendum

Since submitting this article Berlyne, Short, and Vickers (1957) have described two further instances of placental transfer of the L.E. factor. In both cases L.E. cells were demonstrable in mother and foetus. The infants remained healthy, L.E. cells disappearing from the blood by seven weeks.

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A LEUKAEMOID BLOOD PICTURE IN MEGALOBLASTIC ANAEMIA OF THE PUERPERIUM

BY

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Blood pictures simulating leukaemia are known to occur in a variety of conditions, of which acute infections and tuberculosis are the chief. The following three cases are added to the small number already recorded in association with megaloblastic anaemia in the puerperium. They were all admitted to the same obstetric unit, and were encountered within a period of just over three months.

Case Reports

Case 1.—Aged 32. Two previous pregnancies normal. Tiredness, swelling of ankles during last three weeks of third pregnancy. Labour normal, no undue blood loss. Admitted to hospital 10 days after delivery.

Treatment during Pregnancy.—None. During the five days before admission she had taken eight capsules of a compound haematinic, which did not contain vitamin B_{12} but included a minute quantity of folic acid (estimated by the manufacturers as 240 μg. per capsule).

Clinical Condition.—Exhaustion and extreme pallor on admission. Scanty purpuric spots on the abdominal wall, which faded within 24 hours. Intermittent mild pyrexia for five days. A small breast abscess developed on the thirteenth day; this responded rapidly to incision and treatment with "aureomycin." Liver and spleen were not palpable at any stage.

Initial Blood Findings.—Haemoglobin was 2.9 g.%. White cells numbered 23,600 per c.mm. The red cells appeared normochromic and normocytic, though a few nucleated red cells, readily identifiable as megaloblasts, were present in the peripheral blood. Over 45% of the white cells were immature granulocytes, of which many were giant myelocytes and 1.5% were blasts. The mature polymorphs showed hypersegmentation of their nuclei.

Other Investigations.—The sternal marrow on the first day showed megaloblastic hyperplasia. The myeloid series also appeared unduly active, with numerous mitotic figures, but the proportion of blast cells was not increased. Giant myelocytes and metamyelocytes were prominent. Free hydrochloric acid was present in the gastric juice. The faecal fats were normal.

Treatment.—The patient had been admitted during the night, and transfusion of 2 pints of blood was begun almost immediately. The following afternoon folic acid therapy was instituted, beginning with 30 mg. intravenously. For the next three days 15 mg. was given intramuscularly twice a day, and oral folic acid, 10 mg. three times daily, was continued for a further three months. A course of intravenous iron therapy was given from the fifth to the eleventh days, and oral iron substituted thereafter.

Course.—The total white cell count at first fell rapidly, but on the third day it rose again and remained at 20,000 on the fourth and fifth days, the proportion of immature granulocytes reaching 60%. There was a concomitant increase in the number of circulating nucleated red cells. Subsequently, both the total white cell count and the proportion of immature forms gradually fell to normal, the total count reaching 9,900 on the 19th day and the immature cells disappearing by the 75th day. The haemoglobin level, the red cell count, and the packed cell volume all showed a satisfactory response to folic acid therapy. There was a fairly rapid change in the appearance of the red cells in the stained film; the initial normochromic normocytic picture gave way within four days to one of frank macrocytosis, indistinguishable from that of Addisonian anaemia, with a subsequent slow return to normal. The patient was discharged from hospital on the 19th day, and has since attended regularly as an out-patient.

Case 2.—Aged 37. Eight previous pregnancies normal. Admitted to hospital 11 days after premature delivery of ninth child.

Treatment during Pregnancy.—None. Eight tablets of ferrous gluconate before admission.

Clinical Condition.—Marked pallor, with a distinct yellowish tinge on admission. Low-grade pyrexia. No evidence of infection. Liver just palpable; spleen not palpable.

Initial Blood Findings.—Haemoglobin was 3.8 g.% and red blood cells 1.3 millions per c.mm. The white blood cells numbered 10,000 per c.mm., of which 11% were immature granulocytes. The red cells varied considerably in size and shape, but only a few were macrocytic. Intermediate and late megaloblasts were seen in the peripheral blood. Giant metamyelocytes and myelocytes were present, as well as hypersegmented polymorphs.

Other Investigations.—The sternal marrow, on the second day, showed megaloblastic hyperplasia; there was also hyperplasia of the myeloid series, but the proportion of blast forms was not excessive.

Free acid was present in the gastric juice. The faecal fats were normal.

Treatment.—One pint of packed cells was transfused on the first day. Folic acid therapy was begun on the third day, according to the same scheme as for Case 1, but omitting the initial intravenous dose. A course of intravenous iron was begun on the fourth day. Oral folic acid and iron were substituted for parenteral therapy, and were continued until the blood picture had returned to normal.

Course.—The total white cells had risen sharply to 24,500 per c.mm. on the fifth day, 52 hours after the first dose of folic acid, with an accompanying rise in the proportion of immature granulocytes to 25%, and in the number of circulating nucleated red cells. The white cell count reached 26,500 on the sixth day, with 27.5% immature granulocytes, including 1.5% blasts. The total count returned to normal within a few days, and the nucleated red cells disappeared from the blood shortly afterwards, but the immature granulocytes persisted for a further six weeks. By the time the leukaemoid response had reached its height, the red-cell picture had become frankly macrocytic. The anaemia responded satisfactorily to folic acid and iron therapy.

The patient was discharged on the twentieth day. She has attended regularly as an out-patient, and remains well.

Case 3.—Aged 28. Three previous pregnancies; the latter two had been followed by megaloblastic anaemia, treated with folic acid. Megaloblastic anaemia, verified by marrow biopsy, had recurred 16 months after the last pregnancy, and had again responded to folic acid. Myeloid reactions were not observed on any of these occasions. Admitted two hours after delivery of fourth child; labour had been normal, with little blood loss.

Treatment during Pregnancy.—In view of the previous history, folic acid and iron had been prescribed, but had not been taken, during pregnancy. The diet had been extremely poor. Haemoglobin levels only were estimated during pregnancy; they showed a steady fall from 11.0 g.% in the fourth month to 5.5 g.% six days before delivery. Treatment during the last six days comprised only a single injection of vitamin B_{12} and a single tablet of folic acid.

Clinical Condition.—Deathly pale on admission. No pyrexia. No purpura. Liver palpable, one fingerbreadth; spleen not palpable. No evidence of infection.

Initial Blood Findings.—Haemoglobin was 3.1 g.%, and red cells 800,000 per c.mm. The white cells numbered 7,500 per c.mm., including 1.5% immature granulocytes. The stained film showed frank macrocytosis; occasional megaloblasts were seen.

Other Investigations.—Sternal marrow (on day of admission): megaloblastic hyperplasia; white cell precursors not unduly active, but included giant myelocytes. Hypochlorhydria. Faecal fats normal.

Treatment.—Folic acid therapy was begun 10 hours after admission. She received 30 mg. intravenously twice daily for three days, 15 mg. intramuscularly twice daily for three days, and subsequently took 10 mg. by mouth three times daily. A course of intravenous iron lasted from the sixth to the twelfth day. Because of her poor clinical condition, 1 pint of blood was transfused on the seventh day. Oral folic acid and iron were continued after her discharge from hospital on the 17th day.

Course.—On the third day, the total white cell count had risen to 26,000 per c.mm., with 17 to 21% of immature granulocytes, and there was a simultaneous outpouring of nucleated red cells. The total white cell count fell to 18,000 on the fourth day, 12,000 on the sixth day, and 8,500 on the seventh day, but the immature cells persisted, in fluctuating proportions, for a further three weeks. The response of the anaemia to folic acid therapy was satisfactory. Macrocytosis ceased to be evident after two weeks.

Discussion

These three cases of megaloblastic anaemia, presenting in the puerperium, were all associated with a leukaemoid picture in the peripheral blood. In two cases, the leukaemoid reaction was present initially, and became more pronounced within two days of the start of folic acid therapy. In the third, the reaction was negligible initially, but became evident within two days of starting therapy. In all three cases, the total and differential white cell counts gradually returned to normal while the same therapy was continued.

A search of the literature has revealed three cases—two of them fatal—in which a proved megaloblastic anaemia in the puerperium was associated with a myeloid response. That reported by Foy, Kondi, and Murray (1946) followed an abortion; death occurred several months later, and the case was regarded as one of "leukanaemia," though it is quoted by Whitby and Britton (1953) among "authentic cases of pernicious anaemia complicated by myeloid leukaemia." Gillespie and Ramsay (1948) recorded a case which ended

fatally five weeks after delivery; the total white cell count was consistently low, but the differential count included 20 to 50% of myelocytes, promyelocytes, and myeloblasts.

The case reported by Ritchie (1952) bears the closest resemblance to the present series; a severe megaloblastic anaemia in the puerperium was accompanied by an extensive myeloid response, which decreased rapidly over a period of five days, and completely disappeared within three weeks. As in Case 3, the initial total white cell count was normal, with a very few myelocytes and blasts in the peripheral blood, and the outpouring of immature granulocytes was observed two days after institution of folic acid therapy.

The case described by Scott (1948) of a leukaemia-like reaction in the puerperium, associated with a severe anaemia, may have been a further example. The nature of the anaemia was not established, however, and the leukaemoid response was attributed to a single dose of sulphadiazine.

Disturbances of granulopoiesis are probably not uncommon in megaloblastic anaemia of pregnancy, just as they are a usual feature of the blood picture in Addisonian anaemia. According to Callender (1944), "a few myelocytes" are "always present" in megaloblastic anaemia of pregnancy, and Wintrobe (1956) states that, though leucopenia is more frequent than leucocytosis, a few immature forms are often found, as well as multiple segmented neutrophils. The presence of macrogranulocytes in the marrow is considered by Lowenstein, Pick, and Philpott (1955) to be of diagnostic significance.

Heck (1932) observed leukaemoid reactions in association with Addisonian megaloblastic anaemia. He noted that they occurred primarily in cases in which the red cell count was below 2,000,000 per c.mm., and that there might be an increase in the amount of immaturity within a short time of the institution of specific therapy.

The circumstances under which leukaemoid reactions may occur have been elaborately classified by Hill and Duncan (1941), and further considered by Hilts and Shaw (1953). In the present cases, it seems unlikely that any factors other than megaloblastic anaemia and the administration of folic acid were related to the myeloid response. The only clinically evident infection was the breast abscess in Case 1, and this developed only when the myeloid response had almost subsided. No antibiotics or other drugs had been taken, apart from the negligible amounts of haematinics already mentioned.

Considering the present cases and Ritchie's together, it is evident that myeloid responses of varying degree may accompany megaloblastic anaemia of the puerperium, and that they may be temporarily accentuated during folic acid therapy. It is possible that, in all four cases, the association between folic acid therapy and accentuation of the myeloid response was fortuitous, but the close similarity of the time relationships in all four cases makes this highly unlikely. It is difficult to resist the conclusion that folic acid is capable of accentuating the abnormal myeloid response, whether it is already clearly evident, as in Cases 1 and 2, or of very slight degree, as in Case 3 and Ritchie's case.

The part played by folic acid may be explicable in the light of its known effects on white cell development. Marinone and Lourau (1948), studying the effect of folic acid on the bone marrow in rabbits, noted that myelocytes and metamyelocytes increased at the expense of myeloblasts and promyelocytes, and concluded that in the normal animal folic acid acts as a maturation factor for the myeloid cells. The discovery by Farber, Diamond, Mercer, Sylvester, and Wolff (1948) of the beneficial, if temporary, effect of folic acid antagonists on acute leukaemia was prompted by the observation that administration of folic acid conjugates to children with acute leukaemia resulted in an "acceleration phenomenon," demonstrable in the marrow and viscera at necropsy. Rapid haematological and clinical relapse followed the trial of folic acid in chronic myeloid leukaemia (Heinle and Welch, 1948), and haematological improvement accompanied the withdrawal of folic acid. Such observations are in keeping with Swendseid, Bethell, and Bird's (1951) finding that in acute leukaemia rapidly proliferating leucocytes have a content of folic acid approximately five times that of the normal leucocyte.

It would appear that the present cases represent something analogous to Farber's "acceleration phenomenon," occurring not in leukaemia but in a transient leukaemoid reaction accompanying megaloblastic anaemia.

Lastly, the absence of macrocytosis in Case 1, when first seen, deserves comment. It is recognized that the megaloblastic anaemia of pregnancy is not necessarily a macrocytic anaemia; it may be normocytic or even microcytic (Lillie, Gatenby, and Moore, 1954; Lowenstein *et al.*, 1955). Scott (1954) noted that in such cases after a few injections of intravenous iron the red cells tended to become macrocytic, and typical megalo-

blasts could be seen in the blood films; she regarded this phenomenon as so constant as to be a useful aid to diagnosis when sternal punctures could not be carried out. Intravenous iron, however, cannot be implicated in Case 1, as the conversion of a normocytic to a macrocytic picture occurred before the first dose of iron was administered. Unless folic acid played a part in this also, the more probable explanation is that the megaloblastic change in the bone marrow had occurred only a short time previously, and was not clearly reflected in the peripheral blood for several days.

Summary

Three cases are presented in which megaloblastic anaemia, first diagnosed in the puerperium, was accompanied by a leukaemoid blood picture of myeloid type. All recovered on folic acid and intravenous iron therapy. The probable part played by folic acid in accentuating the myeloid response is discussed.

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Rh ANTIBODIES AND THE PROZONE PHENOMENON

BY

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In 1942 Taylor, Race, Prior, and Ikin reported an anti-Rh serum which failed to agglutinate Rh-positive cells in saline suspension except when diluted. They thought that this prozone was due to antigen and antibody not being present in optimal proportions, and that the mechanism was similar to that of the zones that occur in precipitation reactions.

Wiener (1944) described incomplete Rh antibodies and their blocking effects, and considered that this type of antibody might be responsible for the prozones seen with Rh antisera.

Subsequently, various other workers observed prozones with anti-human globulin sera, and Hubinont (1951) considered that these were due to lack of optimal proportions between antigen and antibody.

Van Loghem (1950) also investigated the prozones of antiglobulin sera and considered that they might be due either to the presence of blocking antibodies or to overdosage by excessive amounts of agglutinin, i.e., lack of optimal proportions.

Van Loghem, Kresner, Coombs, and Fulton Roberts (1950) considered these two alternative explanations further, and described experiments to show that excess of agglutinin was the cause of the prozone in the particular antiglobulin reagent which they were studying. They suggested that, when agglutinin is present in high concentration, all the available antigen sites on the red cell surface become occupied by molecules of agglutinin, so that none are free to combine with molecules of agglutinin already attached to other cells, and the lattice necessary for agglutination cannot be formed. This was demonstrated by the fact that washed cells from the prozone would agglutinate when mixed with a further supply of cells bearing free antigen receptors. Cells from the prozone also agglutinated on the addition of suitable concentrations of human γ globulin. The conclusion drawn from these experiments, that the prozone, in this particular case at any rate, was caused by excessive amounts of agglutinin and not by incomplete antibody, appears at first sight to be irrefutable. Nevertheless, certain considerations led us to suppose that this conclusion might be incorrect and that the same phenomenon might occur even though the cause of the prozone was incomplete antibody. The first experiment described below shows that that is so, at any rate in the case of Rh antibodies. The second experiment shows that in some cases even cells from the first negative tube of a titration will agglutinate on the addition of more cells with free receptors.

Experiment 1

Table I shows the results obtained on titrating an agglutinating and an incomplete serum, and a mixture of these with a 3% saline suspension of washed R_2r (cDE/cde) cells at 37° C. The serum Ho. is a saline agglutinating anti-D serum and shows no signs of prozone. The serum Po. is an incomplete anti-D serum and shows no agglutination at any dilution. The third line of the table shows that a prozone is obtained when a mixture of 1 part serum Ho., 4 parts serum Po., and 3 parts saline is titrated. We consider that this particular prozone is due to the presence of incomplete antibody.

TABLE I

TITRATIONS OF ANTI-D SERA WITH R_2r (cDE/cde) CELLS

Serum	Reciprocals of Dilutions										
	1	2	4	8	16	32	64	128	256	512	1,024
Ho. ..	v	v	v	v	v	v	+++	+++	+	–	–
Po. ..	–	–	–	–	–	–	–	–	–	–	–
Mixture*	–	++	+++	+++	++	+	–	–	–	–	–

* Mixture = 1 vol. Ho., 4 vol. Po., 3 vol. saline. v, +++, ++, +, = Degree of agglutination. – = No agglutination.

In the second part of the experiment a further volume of a 3% saline suspension of washed R_2r (cDE/cde) cells was again incubated with one volume of a mixture of 1 part Ho., 4 parts Po., and 3 parts of saline. After one and a half hours' incubation at 37° C., the cells were washed three times in saline, restored to a 3% suspension in saline, and divided into a number of tubes. An equal volume of a 3% suspension of washed R_2r (cDE/cde) cells was added to the first tube, washed rr (cde/cde) cells to the second tube, a volume of undiluted Ho. to the third tube, and a volume of saline to the fourth tube. Saline from the last washing of the cells of the prozone was simultaneously tested with R_2R_2 (cDE/cDE) cells in order to show that there was no free agglutinin in the supernatant. The mixtures were incubated for a further one and a half hours at 37° C. The results of this experiment are shown in Table II,

TABLE II

REAGENTS ADDED TO WASHED CELLS OF PROZONE (1 in 1 dilution of mixture in Table I)

Reagents Added to Washed Cells of Prozone	Result
R_2r (cDE/cde) cells	++
rr (cde/cde) "	−
Serum Ho.	++
Saline	−
Last washing tested with R_2R_2 (cDE/cDE) cells	−

and it can be seen that the cells from the prozone agglutinated on the addition of a further quantity of washed R_2r (cDE/cde) cells, and also on the addition of undiluted Ho., but there was no agglutination in the controls.

Experiment 2

Table III shows the serum Ho. titrated with a washed suspension of $R_1^{u}r$ ($CD^{u}e$/cde) cells. As expected, the titre is much less than with R_2r

TABLE III

TITRATION OF SALINE AGGLUTINATING ANTI-D SERUM WITH D^{u} CELLS

Serum	Reciprocals of Dilutions								
	1	2	4	8	16	32	64	128	256
Ho.	v	+++	+++	+++	+++	+	−	−	−

(cDE/cde) cells (see Table I), and there is no agglutination at a dilution of 1 in 64. In the second part of this experiment a suspension of the $R_1^{u}r$ ($CD^{u}e$/cde) cells was again treated with an equal volume of Ho., diluted 1 in 64, for one and a half hours at 37° C. and, after washing, was mixed with a volume of washed R_2R_2 (cDE/cDE) cells, washed rr (cde/cde) cells, saline, and undiluted Ho., and again incubated, as in Experiment 1. The last washing was also tested with R_2R_2 (cDE/cDE) cells, as before. The results of this experiment are shown in Table IV,

TABLE IV

REAGENTS ADDED TO WASHED CELLS OF FIRST NEGATIVE TUBE IN TITRATION OF TABLE III

Reagents Added to Washed Cells at End of Titration	Result
R_2R_2 (cDE/cDE) cells	++
rr (cde/cde) "	−
Serum Ho.	v
Saline	−
Last washing tested with R_2R_2 (cDE/cDE) cells	−

and it can again be seen that the cells agglutinate on the addition of a further supply of antigen receptors and on the addition of undiluted Ho., but there is again no agglutination in the control tubes.

It might be argued that in this and the preceding experiment agglutinin was eluted from the cells during the second period of incubation and that this eluted agglutinin was responsible for the agglutination of the cells added. In view of this, both experiments were repeated substituting immediate centrifugation and reading of results for the second period of incubation. The results obtained were the same as before, though the agglutination was a little weaker since low temperatures and short times are unfavourable to the action of Rh antibodies.

Discussion

Two explanations have been suggested to account for zoning in agglutination systems. One explanation is that the incomplete antibody is more avid than the agglutinin, and combines preferentially with the antigen, blocking it against the action of the agglutinin. In the high dilutions of a titration, however, only the agglutinin is effective. The second explanation is that the prozone is due to agglutinin excess, i.e., lack of optimal proportions as in the case of precipitation systems. According to this explanation the concentration of agglutinin in the prozone is so great that the antigen receptors of each cell become saturated with molecules of agglutinin and none are free to combine with molecules of agglutinin already attached to other cells. The two hypotheses are not, of course, mutually exclusive.

In their first experiment, van Loghem *et al.* used an antiglobulin reagent showing a prozone with cells sensitized by incomplete anti-D. Cells from the prozone were washed and found to agglutinate on the addition of more sensitized cells. This was considered to show that the prozone in this case was caused by excess agglutinin rather than by incomplete antibody.

Their second experiment showed that washed cells from the prozone would agglutinate on the addition of suitable concentrations of γ globulin. The principle in this case does not appear to differ

from that involved in their first experiment. In each case the cells agglutinate on the addition of more antigen.

Now in our first experiment the prozone was evidently caused by incomplete antibody, since it occurred on the addition of incomplete antibody to an agglutinating serum which showed no prozone itself. Moreover, it can hardly be contended that the prozone in this case was due to excess agglutinin since addition of more agglutinin caused the cells to agglutinate.

The phenomenon of agglutination of cells from the prozone on the addition of more cells with free antigen would be expected, as van Loghem *et al.* suggest, if the prozone were due to agglutinin excess. However, it appears from our experiments that it also occurs when the prozone is due to incomplete antibody, so that this phenomenon cannot be used as an argument in favour of either mechanism.

Nevertheless, it is not easy to see why this phenomenon should occur when the prozone is due to incomplete antibody. Assuming the correctness of the lattice hypothesis of agglutination due to bivalent agglutinin molecules, agglutination would appear to depend not on how many molecules of agglutinin each cell takes up, but on how many molecules become attached to two cells. We think of agglutination as taking place in two stages. In the first stage the cells are taking up molecules of agglutinin free in the serum, and in the second stage they are taking up molecules already attached to other cells, and are, therefore, agglutinating. The two stages may well be concurrent.

This concept seems to us to be fundamental to an understanding of the subject and is supported by the fact that the length of the prozone is dependent on the spatial relationship of the erythrocytes during the reaction. If the cell suspensions and serum dilutions are mixed, centrifuged immediately, and then incubated at 37° C. the length of the prozone is diminished ; whilst if the tube is repeatedly tapped during incubation and centrifuged afterwards, zoning is increased. It seems that in the first case the close contact of the cells during the reaction promotes the second stage, whilst in the second case the reverse occurs.

The amount of antibody which a cell will take up depends not only on the concentration of antibody present, but also on the amount of antibody which it has already taken up. This is a consequence of the quantitative behaviour of antigen-antibody reactions, and means that a cell whose antigen receptors are free from antibody will take up agglutinin more readily than one whose receptors are already partially occupied by antibody. In the second stage of the reaction agglutinin already attached to one cell is seeking attachment to a second cell, and it can be seen that it will more readily attach itself to a cell with free antigen receptors than to one whose receptors are already partially occupied by other molecules of agglutinin and incomplete antibody. Hence it might happen in a prozone that agglutinin molecules which had already attached themelves to a first cell would be unable to join on to a second in sufficient numbers for agglutination to occur, owing to the presence of other antibody molecules already attached to the second cell. They would, however, readily attach themselves to a cell free from antibody.

Experiment 2 illustrates these ideas further. The D^u cells in this case have a reduced ability to take up agglutinin as compared with normal D cells. In the first negative tube of the titration there is no agglutination because the second stage of the reaction has failed. There is, however, enough agglutinin on the cell surface to react with the more potent antigen of R_2R_2 (cDE/cDE) cells. The importance of this in the present connexion is that this is another case where agglutination occurs on the addition of cells with free antigen receptors. Moreover, it could not possibly be contended here that the absence of agglutination in the first place was due to the presence of excessive amounts of agglutinin.

Such considerations show how the phenomenon of agglutination of cells from the prozone on the addition of cells with free antigen receptors can be accounted for whether the prozone is due to agglutinin excess or to incomplete antibody. They also lead to the supposition that there is no real difference between these two causes of zoning. Both, presumably, are due to "antibody excess," that is, to antibody on the cell surface interfering with the second stage of the reaction.

Now the second stage is favoured by a high concentration of agglutinin at the cell surface, since there is then a large number of agglutinin molecules seeking attachment to a second cell. On the other hand, it is resisted by a high concentration of antibody on the cell surface, since this reduces the ability of the cell to take up agglutinin already attached to another cell. It does not matter in this connexion whether the cells have taken up agglutinin alone or both incomplete antibody and agglutinin in the first stage of the reaction ; either will have the effect of reducing the ability of the cell to take up agglutinin in the

second stage. In short, agglutination is promoted by high concentrations of agglutinin at the cell surface but is resisted by high concentrations of antibody (agglutinin or incomplete).

The effect of increasing the concentration of agglutinin is thus twofold. On the one hand it tends to increase agglutination, and on the other hand, by occupying the red cell surface, it interferes with the second stage of the reaction and so tends to prevent agglutination. With very high concentrations of agglutinin it may be supposed that the second effect predominates and a prozone due to pure agglutinin excess is formed.

If incomplete antibody is present as well as agglutinin the sequence of events may be the same as with agglutinin alone, but zoning will occur more readily, since the only effect of the incomplete antibody is to combine with some of the antigen and interfere with the second stage of the reaction.

The crucial question is whether the concentration of agglutinin at the surface of a cell outweighs the blocking effect of the total antibody at the surface of a second cell to which the agglutinin is seeking attachment. Incomplete antibody merely increases this blocking effect, and the blocking effect is the same whether it is caused by agglutinin alone or by incomplete antibody and agglutinin together. In short, zoning can be attributed to the blocking effect of antibody excess.

Furthermore, the cells are not blocked in any absolute sense, but only blocked against a given concentration of agglutinin, since a higher concentration of agglutinin could overcome the blocking effect. This is why cells from the prozone of Experiment 1 will agglutinate in the presence of undiluted agglutinating serum.

Whether or not this explanation of these phenomena is correct does not, of course, affect the validity of the experimental findings, nor detract from the conclusion that agglutination of cells from the prozone on the addition of cells with free antigen receptors is no evidence that the original absence of agglutination was due to agglutinin excess. It is, however, a phenomenon which is very helpful in attempting to understand the mechanism of zoning.

Summary

Washed cells from a prozone caused by incomplete anti-D were found to agglutinate on the addition of more D-positive cells, and also on the addition of more anti-D agglutinin. Washed cells from the first negative tube of a titration of a saline agglutinating anti-D serum with D^u cells were found to agglutinate in the same circumstances. These results show that the phenomenon of agglutination of cells from a prozone on the addition of more cells bearing free antigen receptors cannot necessarily be taken to indicate that the prozone was caused by agglutinin excess.

The phenomenon of zoning appears to be due to excessive amounts of antibody at the cell surface interfering with the second stage of the agglutination reaction, in which agglutinin already attached to one cell is seeking attachment to another.

These considerations lead to the supposition that there is no essential difference between zoning caused by incomplete antibody and that caused by "agglutinin excess." Both are caused by "antibody excess" in the second stage of the reaction.

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THE INFLUENCE OF AGE ON RED CELL DIAMETER

BY

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During the examination of stained blood films from hospital patients, the impression was gained that the red cells tended to be larger in old age than in young or middle life. To investigate this possibility, halometer readings were made on blood films from patients of different ages, and enough evidence was collected to show that there were statistically significant differences between various age groups.

There is very little information about the effect of age on mean red cell diameter (M.C.D.). Saragea (1922) measured the red cell diameters of people in all the decades of age up to 90, but since only one person was examined for each decade the results were not of much value. Price-Jones (1929) did not demonstrate any significant difference with age. Among 100 normal persons for whom he calculated M.C.D.s, all but five were below the age of 60, and all but 10 were below the age of 50. The mean reading for all the 10 persons aged over 50 was 7.137 μ, which is less than the mean for the whole series (7.202 μ).

Olbrich (1947) recorded a number of haematological values in old people, and made Price-Jones curves of blood films from 23 men and 26 women over the age of 64. He compared the figures with those of 10 healthy young persons aged 20–30. The mean of the M.C.D.s for the older age group was 7.6 μ (S.D. 0.187 μ, range 7.1–8.0 μ), and that for the younger age group was 7.03 μ (S.D. 0.1611 μ, range 6.84–7.33 μ). No significant sex difference was found. Although Olbrich's figures suggest a marked increase of diameter in the elderly, the numbers were too small to show the trend in different decades.

Material and Methods

Dry, unstained blood films were obtained by finger prick from 25 males and 25 females in each decade of age from the first to the ninth. In the first decade, children below the age of 1 were excluded on account of the well-known macrocytosis of infancy.

The subjects were in-patients and out-patients of the United Oxford Hospitals and the Nuffield Orthopaedic Centre, and, in the second and third decade, some healthy doctors, nurses, and laboratory technicians were included. Most of the older persons were in-patients or out-patients of the Cowley Road Hospital, Oxford.

No selection was exercised except that persons known to be severely anaemic or suffering from blood diseases were excluded. Bone marrow was examined in cases with an M.C.D. of 8 μ or over, and those cases in which it was abnormal were excluded. One reading was taken from each film, using a Waterfield monochromatic halometer. All readings were made by the same observer (A. I. S.), who did not know the source of the film at the time it was read.

Results

The means of the M.C.D. readings for males and females in each decade are given in Table I.

TABLE I

MEANS OF HALOMETER READINGS (IN MICRONS) FOR DIFFERENT SEXES AND AGE GROUPS

Age	Male	Female
1– 9	6·936	7·084
10–19	6·968	7·132
20–29	6·984	7·120
30–39	6·968	7·076
40–49	7·188	7·212
50–59	7·200	7·112
60–69	7·268	7·264
70–79	7·380	7·348
80–89	7·348	7·436

Up to and including the fourth decade (ages 30–39) there is no apparent change with age, although the curve for females lies consistently above that for males. The mean for the four decades is 7.03 μ. From the fourth decade onwards there is a steady rise in the means, with a regression coefficient of 0.06 μ for each decade.

Table II and Fig. 1 show three distribution curves constructed from the three age groups 1–29, 30–59, and 60–89. In each of these groups there is a wide scatter, the standard deviations being 0.28, 0.28, and 0.27 μ respectively; but it will be

TABLE II
NUMBERS OF CASES FALLING IN THREE AGE GROUPS, CLASSIFIED ACCORDING TO M.C.D. IN MICRONS

μ	No. Persons Aged 1–29	No. Persons Aged 30–59	No. Persons Aged 60–89
8·0 }	2	1	5
7·9 }			
7·8 }	0	3	11
7·7 }			
7·6 }	3	14	39
7·5 }			
7·4 }	30	37	35
7·3 }			
7·2 }	43	33	36
7·1 }			
7·0 }	30	34	19
6·9 }			
6·8 }	27	22	5
6·7 }			
6·6 }	15	6	0
6·5 }			
6·4 }	0	0	0
6·3 }			
Total ..	150	150	150

noticed that readings above 7.6 μ are rare below the age of 30, and become common in the older age groups. For instance, in the 80–89 group there were 13 readings above 7.6 μ out of the total of 50 cases.

Discussion

Our figures show a highly significant upward trend in the halometer readings for each decade after the 30–39 age group. It is not claimed that this trend necessarily applies to the population in general, as the increased values in elderly hospital patients could well be the result of diseases which become increasingly common with age. Neither do we place any weight on the absolute values for M.C.D. obtained with the Waterfield halometer. This type of measurement was chosen as the only practical means of obtaining a large enough number of figures for the study. Values obtained with this halometer are fairly reproducible; for instance, five different observers each made 100 readings on a set of 10 slides from different patients (500 readings in all), and the coefficient of variation for random error was only 2.2% (Biggs, personal communication). Inspection of the stained films corresponding to those giving high halometer readings did not show much anisocytosis, and apart from an increase in apparent size the only abnormality was a tendency in some cases to formation of target cells.

Until our findings can be confirmed in a series of persons who are not suffering from any recognizable disease, it would be premature to suggest possible reasons and mechanisms for the rising trend of M.C.D.s with age. The main conclusion to be drawn is one of importance to the practising haematologist, i.e., the red cells of aged patients without evidence of megaloblastic anaemia often show moderate degrees of enlargement which, in the young, would demand thorough investigation to exclude serious disease.

Summary

The mean red cell diameter was estimated in a series of 450 persons, mostly hospital patients, using a Waterfield halometer. Twenty-five males and 25 females were examined from each decade of age up to 90 years old.

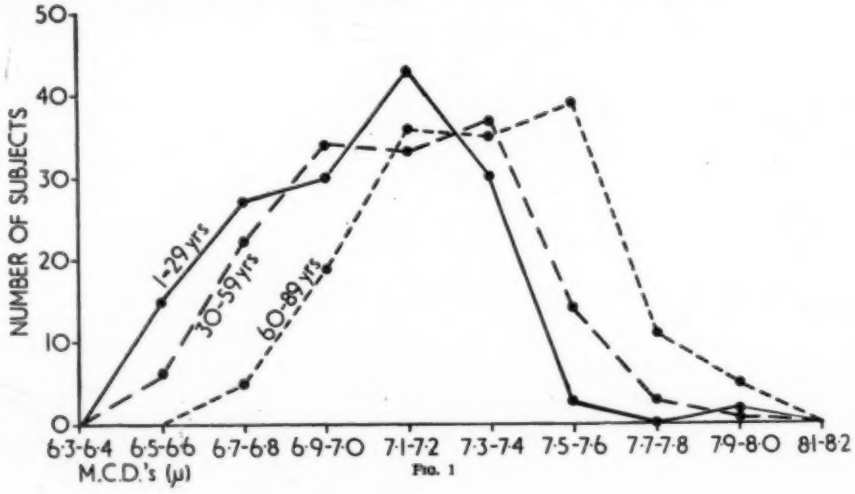

FIG. 1

In the first four decades (ages 1–39) the means of the halometer readings remain the same in each decade, but the level for females is slightly above that for males. In the later age groups this possible sex difference disappears, but there is a significant continuous upward trend reaching in the ninth decade a level of 0.37 μ higher than the mean for the age group 1–39.

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COMPARISON OF QUICK'S, OWREN'S, AND WARE'S TECHNIQUES FOR THE CONTROL OF ANTI-COAGULANT THERAPY

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Anticoagulant therapy is now in widespread use and is generally accepted as being of real value in the treatment of myocardial infarction and other thrombo-embolic diseases. In the last few years long-term continuous treatment with anticoagulants to prevent further attacks of infarction has been more widely adopted. With appreciable numbers of patients, both in-patients and out-patients, receiving anticoagulant drugs, it is perhaps timely to evaluate the different methods used for the control of anticoagulant therapy.

Until fairly recently Quick's method was almost universally employed and considered as the simplest and best means of achieving laboratory control of anticoagulant therapy. Even to-day it is still the method most widely used. More recently, however, Owren's "P and P" (prothrombin and proconvertin) technique (Owren and Aas, 1951) has been advocated as being more sensitive and reliable, and it is claimed that with its use there is a lower incidence of haemorrhagic manifestations. Ware and Stragnell (1952), using a modified version of Owren's technique, found that it gave better results than Quick's method.

In the New End Hospital anticoagulant unit, Quick's, Owren's, and Ware's methods were used simultaneously to control anticoagulant therapy of in-patients over a period of several months, and in all 800 blood samples were tested by all three techniques. It was found that, apart from the method of preparation of the prothrombin- and proconvertin (factor VII)-free ox plasma, Owren's method was better than Ware's.

When performing the test by Ware's method, the test plasma is diluted with water and only half as much calcium and thromboplastin is added as is used of the other reagents. As a result the difference between the minimum and maximum therapeutic clotting times is much smaller than with Owren's method. In practice, however, this shorter time is less convenient, especially when large numbers of tests are being made. Again, below a level of 10% there was sometimes a marked lack of correlation between the two methods with, on the whole, more reliable results with Owren's technique.

After this preliminary trial with all three methods, a further comparison was then made between Quick's and Owren's methods. In all, 2,100 blood samples were tested with both techniques over a period of 16 months for the control of in-patients on anticoagulant treatment. As a result of this trial there appeared to be no doubt that Owren's method had one decided advantage, i.e., it reflected changes in the "prothrombin" level ("prothrombin" being taken as meaning both prothrombin and proconvertin, both of which are affected by coumarin drugs) 24, or even more, hours earlier than did Quick's method. Quick's method seemed to lag behind Owren's. The great advantage with Owren's method was therefore that one was able to make the necessary adjustments in the dosage of the anticoagulant drug more quickly and easily. This meant that the correct maintenance dose of the anticoagulant was more quickly arrived at and lessened the number of blood tests required for control of the treatment.

For treatment of an acute illness it is necessary to bring the patient under therapeutic control as quickly as possible. In these circumstances, when frequent and big changes in the dose of the anticoagulant drug are usually necessary, and when it is possible to take fairly frequent blood tests, Owren's method does enable one to adjust the dose more rapidly and easily. One point must, however, be emphasized. It has been said that because Owren's method is more sensitive than Quick's it enables a more accurate estimate to be made of the dose of the anticoagulant drug required. This is not strictly true. Quick's test is just as "sensitive" to the effects of small changes in the dosage

of anticoagulant drugs, but it takes longer for these effects to be noticeable. Therefore, with long-term anticoagulant therapy, when blood tests are made every few weeks, or even at longer intervals, it is difficult to see how Owren's technique will materially improve on Quick's method. Indeed, in a busy out-patient clinic, where large numbers of tests have to be made quickly to avoid keeping the patients waiting too long, the speed and simplicity of Quick's method are a decided advantage. Experience with Owren's method for out-patient therapy in this unit is, however, too limited for a definite conclusion yet to be drawn.

Preparation of Reagents for Owren's Method

For successful results with Owren's technique it is essential to prepare a thromboplastin with a constant and reproducible activity and a reliable prothrombin and proconvertin-free ox plasma. Unless these two reagents give constant reproducible clotting times, the results with Owren's method will be most unsatisfactory. Only familiarity with the preparation of these reagents will give the desired results, but the methods of preparation outlined below have been found to be reliable. In the main they are very similar to Owren's instructions, but minor details are worth emphasizing.

Thromboplastin. — As advocated by Owren and myself for many years, for Quick's technique a saline extraction of human brain is used. For Quick's test I have always felt that a saline extraction of brain gives better and more reproducible results than an acetone-dried brain extract. Owren for his technique says, "A thromboplastin with a constant and reproducible activity is not obtained by extraction of dried brain substance." With this I fully agree. It must be stressed that for Owren's method, as for Quick's, the first essential for reliable results is a potent thromboplastin which gives constant reproducible activity. The following method is used to prepare the thromboplastin:

A whole human brain is used. It is washed under tap water and freed of membranes and blood vessels. Any brain tissue with obvious blood is discarded. The brain is then emulsified by a mechanical homogenizer for about 60 seconds to produce a fine emulsion. For this emulsification 2½ to 3 litres (Owren uses 1½ litres) of normal saline (0.9 g. sodium chloride per 100 ml. distilled water) heated to 45° C. to 50° C. is used. This fine emulsion is next centrifuged for 15 minutes only at 2,000 r.p.m. to remove all the coarser sediment. The supernatant fluid is poured off; it does not matter at this stage if a little sediment of brain tissue is decanted over into the supernatant fluid. The supernatant extract is incubated at 37° C. for two hours, no longer. During the period of incubation the brain extract should be thoroughly shaken for several minutes on three or four occasions.

After two hours' incubation the brain extract is centrifuged for a second time at 2,000 r.p.m. for about 18 to 20 minutes. After centrifuging it will be found that there is a small sediment of brain tissue present. The supernatant fluid is very carefully poured off, making sure this time that no sediment is decanted over. This point is essential, as in the final brain extract there must be no sediment, visible to the naked eye, unless the extract has been standing for many hours. This final brain extract is now ready to be standardized and tested for activity. It must give a Quick prothrombin time of 11 to 13 seconds, ideally 12 seconds, with pooled normal control plasma. If the brain is too concentrated it may have to be diluted, as Owren points out, to optimum strength. This means the least concentrated brain extract which will give a Quick time of 11 to 13 seconds. The following procedure for standardization has been found to give reliable and reproducible results.

After the incubated extract has been centrifuged and the supernatant fluid decanted, great care being taken not to allow any sediment to spill over, a colorimetric reading is taken. With a red filter (640 wavelength) in a 15 mm. tube (an E.E.L. colorimeter giving a reading of 41 with the E.E.L. permanent opalescent standard is used here) the colorimetric reading should be in the region of 20 to 30. If the reading is below 18 (which is unlikely unless the brain extract has been centrifuged for much longer times than stated above) resuspend the supernatant in the sediment and centrifuge again but this time for a shorter period, say 15 minutes at 2,000 r.p.m., instead of 20 minutes. If the colorimetric reading is between 20 and 30, the *p*H of the supernatant extract is adjusted to 7.35 with N/2 caustic soda. After adjusting the *p*H add one-tenth the volume of Owren's buffer (see below).

A Quick time is now estimated with this extract with normal control plasma and the time should be between 11 and 13 seconds. If the Quick time is above 14 seconds it is advisable to prepare another brain extract. If the Quick time is between 11 and 13 seconds the brain extract is now diluted with buffer-saline, as necessary, to give a colorimeter reading of 18 to 20. (Buffer-saline is made up by adding one volume of Owren's buffer to nine volumes of normal saline.) The brain extract is stored at −20° C. in tightly stoppered tubes. Tubes (3 in. by ½ in.) are convenient to use as they can be put directly into the water-bath to warm up rapidly to the desired temperature of 37° C.

When a fresh brain extract is made up for use, it is perhaps desirable (although not essential for Owren's method) to compare it with the brain extract in current use for control of anticoagulant therapy. Therefore, in addition to making sure that the normal control value is correct, i.e., between 11 and 13 seconds, the plasma of a patient on anticoagulant therapy with a prolonged Quick time should be tested

with the old and new brain extracts, and with both extracts the Quick time should be approximately the same to within a few seconds.

With experience in estimating the optimum concentration of brain extract, the above procedure has given brain extracts yielding constant and reproducible results over the last 18 months. One quickly learns to gauge the correct centrifuging times so as to avoid getting too concentrated or too dilute a brain extract. For Owren's method it is essential not to have too concentrated a brain extract and yet it must be sufficiently potent to give constant and reproducible results.

It is perhaps as well to note the difference between the procedure outlined here for preparing the brain extract and that advised by Owren. These differences are mainly a matter of convenience. Owren uses 1½ litres of normal saline for emulsifying a whole brain, whereas here it has been found much more convenient to use 2½ litres, as less dilution is then necessary at a later stage in the preparation. Again Owren, after emulsification of the brain, allows the emulsified brain to stand at room temperature until the temperature of the extract falls from 50° C. to 20° C. This may, however, take the best part of the day. By doing an initial short centrifuging for about 15 minutes at 2,000 r.p.m. and incubating the supernatant fluid for two hours, it has been found possible to speed up the extraction and still get a sufficiently potent thromboplastin. Finally, instead of adding by trial and error different amounts of buffer-saline to arrive at the optimum concentration, the colorimetric readings quoted above give a very accurate guide to the optimum concentration of the final extract and result in an easily standardized thromboplastin. It is well worth while taking considerable trouble in preparing the brain extracts so as to get as potent and standardized a thromboplastin as possible.

Prothrombin and Proconvertin-free Ox Plasma.—Ware's method for preparation of the prothrombin- and proconvertin-free ox plasma has been found to be the simplest and most reliable. The following technique is used here:

Bovine blood is collected in wide-mouthed jars containing 100 ml. of 2% potassium oxalate for 900 ml. of blood. In collecting the blood it is important to mix well to prevent fine clots. To separate the plasma it is necessary to centrifuge for 30 to 45 minutes at 2,000 r.p.m. Adsorption of the ox plasma is carried out using barium sulphate, 20 to 25 g. for each litre of plasma. (Most of the ordinary commercial preparations of barium sulphate as used for *x*-ray examinations give reliable results.) Adsorption is carried out for 20 to 30 minutes at room temperature, and about every four to five minutes the plasma must be thoroughly shaken to disperse the barium sulphate throughout the plasma. The plasma is then centrifuged for 30 minutes at 2,000 r.p.m. to remove the barium sulphate. The plasma is next filtered once through a Seitz filter, using a 50% asbestos filter pad. One 14 cm. pad is used for each 400 ml. of plasma. As the filtration is very slow, pressure is necessary, usually 10 to 15 lb. per sq. inch.

The plasma is now tested by doing a Quick time, which should be longer than 20 minutes. An additional method of testing to make sure that sufficient prothrombin and proconvertin have been removed is to add an equal quantity of normal serum to the plasma and then do a Quick time, which should be 80 seconds or more.

Finally, the *p*H of the plasma is adjusted to 7.35 by adding N/2 hydrochloric acid, and the plasma is stored in tightly stoppered tubes at −20° C., where it keeps stable indefinitely.

For the collection of bovine blood it is important to note that potassium oxalate (2% or 2.5%) must be used and not citrate, because if citrate is used it is not possible to get a sufficiently prothrombin-free ox plasma by adsorption with barium sulphate. Once the plasma has been thawed out it must not be refrozen. The plasma remains stable at room temperature for four to six hours at least, and probably for most of the day.

Owren, in preparing the ox plasma, uses citrated blood and filters through a 20% asbestos filter pad, following this with a second filtration through 50% asbestos. Here it has been found that with this technique the speed of filtration and the amounts of plasma filtered through each filter pad require constant attention, much more so than with Ware's method. Using Ware's technique invariably a plasma sufficiently free of prothrombin and proconvertin is obtained, whilst at the same time it is much easier and quicker to carry out.

Owren gives as an alternative method adsorption with barium sulphate alone, using 100 g. for each litre of plasma and adsorbing for five minutes. One adsorption with barium sulphate, even using such large amounts, does not always give the desired results and it is often necessary to repeat the adsorption using fresh barium sulphate after removing the first lot of barium by centrifuging for 30 minutes at 2,000 r.p.m. As Ware's technique invariably gives the desired result, as well as being quicker and easier to carry out, here it is always used. It also has the advantage that filtration through a 50% asbestos pad will remove fine particles of barium sulphate that have not been removed by centrifuging. It should be noted that, whereas Ware uses a 40% asbestos filter, a filter pad of 50% asbestos content is recommended here.

Pooled Normal Human Plasma.—To prepare the standard dilution curve and also for checking each day's routine tests, plasma is collected from 10 to 12 normal people. The pooled plasma is stored in small amounts at −20° C. in tightly stoppered tubes. The bulk of the plasma is put up in accurately measured 0.5 ml. amounts suitable for each day's routine tests. A few tubes with 1.5 ml. amounts are stored for preparing the standard curve. There is a very slight diminution in the prothrombin and proconvertin con-

tent of the stored pooled normal plasma over several months, but not sufficient to interfere with the normal routine testing.

Citrate-heparin Anticoagulant.—It is a common finding that, when citrate or oxalate is used for collection of blood specimens, there is often an appreciable shortening of the Quick time after the blood has been standing for a short time. This shortening of the Quick time is supposed to be due to activation of proconvertin: the change is entirely unpredictable and may be very slight or considerable. In 18 to 24 hours the Quick time usually returns to its original value.

Ware devised a special anticoagulant which eliminates this activation of proconvertin. He adds 100 μg. of standard heparin to each millilitre of anticoagulant used, whether oxalate or citrate.

For collection of the blood samples Owren (Owren, 1956) advises against the use of oxalate, as he has found that the calcium-oxalate precipitate adsorbs a small amount of prothrombin and proconvertin. This finding can be confirmed, and here it has been found that the citrate-heparin anticoagulant is preferable to oxalate-heparin, so 3.13% sodium citrate is used.

Owren's Buffer.—This is used for preparation of the thromboplastin and also for making up the diluting fluids for carrying out the actual tests. Sodium diaethyl barbiturate, 11.75 g., sodium chloride, 14.67 g., and 0.1 N. hydrochloric acid, 430 ml., are mixed together: distilled water is then added to bring the total volume up to 2,000 ml. This buffer should have a *p*H of 7.35. It is best stored at 0.5° C. to prevent the growth of moulds.

Preparation of the Standard Dilution Curve

Whenever a fresh batch of reagent, such as the thromboplastin, ox plasma, or fresh pooled control human plasma, is used a new standard curve must be prepared. The more carefully the thromboplastin extracts are standardized by arriving at the optimum concentration of the particle size of brain tissue in the extract, the smaller will be the difference between the standard curves prepared with different brain extracts. For convenience' sake attempts are made to standardize each fresh batch of brain extract with the brain extract last used. Nevertheless, no matter how much care is taken to do this, minor differences will appear with each new batch of brain extract and ox plasma, so that new standard dilution curves must be prepared for each fresh reagent.

Owren strongly advises that the concentration of heparin, citrate, ionic strength, and *p*H should be kept constant in all dilutions made when preparing the standard dilution curve. To do this two dilutions of human plasma are necessary. First, a primary dilution using what he calls diluting fluid I, which is made up by mixing 100 ml. of citrate-heparin anticoagulant and 500 ml. of normal saline. This diluting fluid has the same citrate, heparin, and ionic strength as the normal or test plasma collected in the special citrate-heparin anticoagulant, using 1 vol. of anticoagulant to 9 vols. of blood.

Using diluting fluid I, a primary dilution of the normal pooled control human plasma (collected and stored as mentioned earlier) is made to give 50%, 25%, 12.5%, and 6.25% concentrations of plasma. Each of these dilutions has the same concentration of citrate and heparin and the same ionic strength as the original 100% concentration of plasma. A second dilution is now made from each of the 100%, 50%, 25%, 12.3%, and 6.25% concentrations, but this time a second diluting fluid is used. Owren uses a second diluting fluid because he has found that his method works better when the final plasma dilution has a lower concentration of citrate and heparin than the original specimen of plasma. Owren's diluting fluid II, as he calls it, is made up as follows: mix 200 ml. of Owren's buffer, 200 ml. of 0.754 g. sodium citrate per 100 ml., and 600 ml. of normal saline. In practice it is even more convenient to use a still lower concentration of citrate and heparin in the final dilutions, and excellent results have been obtained by using as a second diluting fluid 200 ml. of Owren's buffer and 800 ml. of 1/5 normal saline (0.18 g. sodium chloride per 100 ml. distilled water).

To prepare the second dilutions, a 1 in 10 dilution is made from each of the 100%, 50%, 25%, 12.5%, and 6.25% concentrations made with diluting fluid I. To do this 0.2 ml. is pipetted from each of these dilutions into 1.8 ml. of the second diluting fluid. Each of these dilutions is then tested by Owren's method and the clotting times plotted on double logarithmic paper: a straight line should be obtained. Owren states that the line tends to "bend upwards" below 5%. It has been found here that no matter how much care is taken in preparing the standard dilution curve the line tends to curve upwards at about 7% or 8%. In practice this is of little significance.

It should be noted that with Owren's method of preparing the standard dilution curve the 100% value for normal plasma corresponds to a final dilution of 10%, the 50% and 25% concentrations correspond respectively to final dilutions of 5% and 2.5%, and so on. This is because with Owren's technique all the actual blood tests are carried out in a dilution of 1 in 10. This method of preparing the standard dilution curve sounds complicated, but with practice it becomes quite simple. All dilutions must be made with great care.

In a fairly busy anticoagulant unit enough brain extract and ox plasma can easily be prepared at one time to last about three to four months; it is thus necessary to prepare a standard dilution curve only once in this period. During the 18 months in which Owren's method has been in routine use in this anticoagulant unit many different brain extracts and ox plasmas have been prepared, and the standard dilution curves do not differ markedly from each other. The 50% dilution of pooled normal plasma usually gives a clotting time of between 35 and 40 seconds with different batches of reagents; the 25% dilution of pooled normal plasma usually gives a clotting time

in the region of 53 to 58 seconds and the 12.5% dilution times of 82 to 94 seconds.

Routine Tests

Before each day's tests of the blood specimens a control test is done to make sure that all reagents have been properly standardized. The 25% dilution of the normal pooled plasma is the most suitable for test purposes. For convenience and speed the bulk of the pooled normal human plasma is stored in accurately measured 0.5 ml. amounts in tubes at −20° C. The plasma is thawed rapidly in a water-bath at 37° C. and then 1.5 ml. of diluting fluid I is added and carefully mixed. This gives a primary dilution of 25%. Then 0.2 ml. of this dilution is added to 1.8 ml. of the second diluting fluid and Owren's test is carried out in duplicate. The clotting times should correspond, within a few seconds, to the times of the 25% concentration on the standard dilution curve.

Having carefully checked the reagents, each plasma is tested in duplicate by Owren's method and the actual clotting times in seconds converted to a percentage from the standard dilution curve. The actual tests are carried out as follows: Thromboplastin, 0.2 ml., 0.2 ml. of ox plasma, and 0.2 ml. of test plasma diluted 1 in 10 with diluting fluid II are mixed together and warmed in a water-bath at 37° C. for at least two to three minutes. Owren's test, like Quick's, or even more so, is very sensitive to minor variations in temperature. It is therefore very important for all reagents and the diluted test plasma to be kept at 37° C. for several minutes. Calcium chloride, 0.2 ml., previously warmed to 37° C., is then added and the clotting time measured. Owren says that calcium chloride of optimum concentration should be added and for each fresh batch of reagents the optimum concentration of calcium chloride must be ascertained. In practice M/30 calcium chloride invariably gives sufficiently accurate results and therefore this strength is always used.

Collection of Blood Specimens

As with all blood clotting studies, it is essential that all the blood specimens should be taken with a minimum of trauma and as quickly as possible. The needle must always be removed from the syringe and the blood at once intimately mixed with the citrate-heparin anticoagulant. All the test plasmas should be carefully examined before testing for the presence of fine clots which would give false results.

For the control of anticoagulant therapy Owren advises a "P and P" level of 10% to 30%. I believe that a lower level is necessary for treatment of acute thrombo-embolic diseases, and I try to maintain all patients at about 10% to 14%. Levels between 6% and 8% should be avoided for any period of time and levels below 5% are hazardous.

Summary and Conclusions

The results of controlling anticoagulant therapy by the simultaneous use of Quick's, Owren's, and Ware's modification of Owren's technique have been compared. All three methods were carried out on 800 test plasmas, and Owren's method was found to be more convenient and slightly better than Ware's. Quick's and Owren's methods were then further compared and, in all, 2,100 test plasmas from in-patients on anticoagulant therapy were tested by both methods over a period of 16 months. There appeared to be no doubt that Owren's technique gave better results for the control of anticoagulant therapy, at least for in-patient, as opposed to out-patient, long-term therapy. Owren's method reflects changes in the blood 24 or even more hours before Quick's method does. Therefore changes in the dosage of the anticoagulant drug can be made more rapidly and the correct maintenance dose thus more quickly determined. In the long run Quick's method will reflect even the most minute change in the dosage of the anticoagulant drug, but it takes longer to do so. For anticoagulant therapy for an acute thrombo-embolic episode, when it is essential to get and maintain the patient under adequate anticoagulant cover as rapidly as possible and where it is usually necessary to make rapid and big changes in the dose of the anticoagulant drug, Owren's method is definitely better than Quick's. For out-patient long-term therapy, however, where the question of urgency does not arise and where blood specimens are taken at intervals of several weeks or more, I have yet to be convinced that Owren's method is superior to Quick's method.

For Owren's method it is essential to prepare an active and potent thromboplastin giving constant and reproducible results. The concentration of the thromboplastin is critical, and both a too concentrated and a too dilute thromboplastin must be avoided. The method outlined above for preparation of a saline extract of human brain has given very reliable and constant results with Owren's technique (and also with Quick's method). A saline extract of human brain keeps stable indefinitely at −20° C. An acetone-dried extract, besides being more troublesome to prepare, does not give such constant results.

For preparation of the prothrombin- and proconvertin-free ox plasma Ware's technique, using adsorption with barium sulphate followed by filtration through a 50% asbestos filter pad, gave better results than Owren's and it is also much quicker and simpler to carry out.

Better results were obtained by using a lower concentration of citrate and heparin and a lower ionic strength for dilutions of the test plasma than advised by Owren. The addition of heparin to the

anticoagulant for collection of the blood specimens does, as pointed out by Ware, stabilize the clotting factors for several hours at least, presumably by preventing the activation of proconvertin. As observed by Owren, the calcium-oxalate precipitate does adsorb small amounts of prothrombin and proconvertin, so it is best to use citrate-heparin and not oxalate-heparin to collect the blood specimens.

In carrying out the actual test the diluted test plasma, thromboplastin and ox plasma must be warmed together for at least two to three minutes at 37° C., as minor variations in temperature can have significant effects.

The thromboplastin, ox plasma, and pooled human control plasma all keep stable for many months, at least three to four, when stored at −20° C. in tightly stoppered tubes. The thromboplastin and ox plasma remain active and stable at room temperature for four to six hours.

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OWREN'S METHOD FOR THE CONTROL OF ANTICOAGULANT THERAPY

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Anticoagulant therapy is a well-established treatment for coronary thrombosis, pulmonary embolus, and other thrombotic or embolic episodes. The anticoagulant drugs used are usually those of the dicoumarol group. It is known that this group of drugs, "dindevan," "tromexan," "marcoumar," etc., depress the production of prothrombin and possibly to a greater extent that of factor VII (proconvertin). There is a variable time lag between dosage with the particular drug and its effect on the prothrombin and factor VII levels. It is this time lag and the method of estimating the level of the appropriate coagulation factors that have led to some difficulty in controlling the dosage of anticoagulant to be used in individual patients. The Quick one-stage method or some modification of this test has usually been the method of choice in this country. The two-stage method has also been used. The one-stage methods are sensitive to changes in factors V and VII levels and to a lesser extent of prothrombin; in the two-stage methods factor VII levels are ignored, and this latter fact is of importance, as it is known that factor VII is more markedly reduced than prothrombin. In 1951 Owren and Aas introduced the so-called P and P method (prothrombin and proconvertin) for the control of anticoagulant therapy. The method is sensitive to changes in prothrombin and factor VII levels, but insensitive to changes in factor V and fibrinogen. The method is basically a one-stage one with the following modifications.

(1) The plasma is diluted to increase sensitivity, and this dilution, of course, also minimizes the effect of any heparin in the patient's plasma. (2) Bovine plasma from which the prothrombin and factor VII have been adsorbed is added as a source of fibrinogen and factor V. (3) A standardized saline extract of human brain is used as the thromboplastin. (4) Ionic strength is kept constant by the use of two diluting fluids.

The advantages of this method have been stressed by Owren and Aas (1951), by Astrup, Müllertz, and Hansen (1951), by Müllertz and Storm (1954) (who maintained anticoagulant therapy during major surgery), by Astrup and Müllertz (1954), and by Owren (1954). The advantages claimed by these authors have been: (i) Greatly increased sensitivity over Quick's method, especially in the near normal range, making it very much easier to judge the initial hypocoagulability and subsequent dosage. (ii) There is less random variability than in the unmodified test because irrelevant factors are supplied in standard form. This is very important for the accurate stabilization of patients who will be receiving long-term anticoagulant therapy as out-patients. (iii) The method measures the combined effect of prothrombin and factor VII, both of which are important and which may be reduced to a different extent at different stages of treatment. (iv) In Quick's method the optimum calcium concentration may not be used, owing to variations in haematocrit values. This effect is minimized by the dilution. (v) The method is unaffected by small amounts of heparin in the patient's plasma. (vi) The use of a small amount of heparin in the anticoagulant used for the collection of the patient's blood sample enables accurate estimations to be made on blood samples which have been sent through the post. This is also advantageous in the long-term treatment of out-patients.

On the evidence of these observations it was decided that the method should be investigated in this department.

Reagents

The method given here is the one used routinely in this department and differs slightly from Owren's original method.

A. Stock Solutions.—The following are required:

Owren's Buffer.—Take 5.878 g. sodium diethylbarbiturate, 7.335 g. sodium chloride, and 215 ml. 0.1 N hydrochloric acid, and make up to 1 litre with distilled water. The resultant solution should be of *p*H 7.33.

Citrate Solution.—This is 31.3 g. trisodium citrate (dihydrate) made up to 1 litre with distilled water.

25.6 Molar Trisodium Citrate.—For this 240 ml. 3.13% trisodium citrate solution is made up to 1 litre with distilled water.

One Tenth Molar Calcium Chloride.—See Biggs and Macfarlane (1957).

B. Diluting Solutions.—The first (sol. I) consists of 100 ml. 3.13% trisodium citrate solution made up to 700 ml. with 0.9% sodium chloride solution. (This diluting fluid is used only for serial dilutions of pooled normal plasma, before diluting 1 in 10 with diluting fluid B.)

The second (sol. II) contains 200 ml. Owren's buffer and 200 ml. 25.6 M. molar trisodium citrate solution made up to 1 litre with 0.9% sodium chloride solution. (This diluting fluid is used for the 1 in 10 dilution of patient's and normal plasma.)

C. Anticoagulant.—This is made up of 250 ml. 3.13% trisodium citrate solution, 25 mg. heparin, and 25 mg. merthiolate. All the above solutions should be stored at 4° C. when not in use.

D. Brain Extract.—A whole fresh human brain is freed of all blood vessels and membranes. The brain matter is emulsified in 1,500 ml. of 0.9% sodium chloride solution which has previously been warmed to a temperature of 37° C. The emulsification of the brain is best carried out with a "blending machine." We have found the M.S.E. "Ato-mix" very satisfactory for this purpose. The resultant emulsion is centrifuged at 1,800–2,000 r.p.m. for 30 minutes, the supernatant fluid is decanted, the deposit being discarded. Dilutions of a small sample of the supernatant fluid are made in 0.9% sodium chloride solution. The following dilutions are usually sufficient: 1/1, 1/2, 1/4, 1/8. The dilutions are used as thromboplastin in an ordinary one-stage test with normal plasma and M/40 calcium chloride. The bulk of the supernatant fluid is then diluted to correspond with the trial dilution giving the shortest one-stage time. A satisfactory preparation gives one-stage clotting times of 13 to 16 seconds. Finally 10% of Owren's buffer is added to the brain extract. The extract is then distributed into suitable containers and stored at −20° C. The most suitable container for this purpose (and for the bovine plasma, see below) will obviously depend on the requirements of the individual department. We have found that a 5 ml. screw-capped Bijou bottle contains sufficient thromboplastin for the number of tests we have to do on a normal day. It is imperative that a fresh bottle of thromboplastin should be used each day, and that once a bottle has been thawed it should not be refrozen for use at a later date.

E. Bovine Plasma.—Bovine blood is collected at the slaughterhouse into 3.5% aqueous potassium oxalate in the proportion of 1 part of oxalate solution to 9 parts of blood. After thoroughly mixing, the blood is centrifuged to remove the cells. To each 100 ml. of plasma is added 2 g. of citrate-washed barium sulphate (Biggs and Macfarlane, 1957). The barium sulphate plasma suspension is gently agitated for 15 to 20 minutes. At the end of this time the barium sulphate is removed by centrifuging. A second adsorption and very occasionally a third adsorption with similar amounts of barium sulphate is necessary. The plasma should be tested after the removal of the second addition of barium sulphate. A mixture in equal parts of absorbed plasma, brain extract, and calcium chloride should take more than 30 minutes to clot at 37° C. The clotting time of a similar mixture to which serum diluted 1 in 10 has been added should exceed 90 seconds. Citrated plasma diluted 1 in 10 and added to the mixture should shorten the coagulation time to 40 seconds or less. A coagulation time much greater than 40 seconds probably indicates over-adsorption and will lead to unnecessarily long coagulation times, making the method laborious and time-consuming. The adsorbed plasma is finally stored in a similar manner to the brain extract at −20° C. In this department 5 ml. amounts are put up in Bijou bottles. It is essential that the plasma is stored in approximately equal amounts.

Technique

A. Details of the Method.—Blood is collected into the heparin-citrate anticoagulant in the proportion of 1 part of anticoagulant to 9 parts of blood. The plasma is obtained by centrifuging and subsequently diluted 1 in 10 in diluting fluid II. Amounts of 0.2 ml. of plasma and 1.8 ml. of diluting fluid are suitable for routine use. Two portions, each of 0.1 ml. of diluted plasma, are placed in each of two 2½ in. × ⅝ in. test tubes, and 0.1 ml. of thromboplastin and 0.1 ml. of bovine plasma are added to each tube. The tubes are then placed in a water-bath at 37° C. for a minimum of three minutes up to a maximum of six minutes. To each tube is added 0.1 ml. of previously warmed $CaCl_2$ solution of appropriate concentration. The time taken for the mixture in the tubes to coagulate is recorded. The coagulation time of a normal sample by this method is usually between 30 and 40 seconds. and plasma samples from patients on anticoagulant therapy may take up to perhaps 200 seconds to clot. It is obvious that recalcifying single test samples is an extremely tedious task. It has been found convenient in routine use to recalcify six or even eight tubes in quick succession if sufficient stop-watches are available. The organization required for this multiple testing is now described.

Enough brain and bovine plasma is pipetted out for about 24 tests. Diluted plasma samples from three patients are pipetted out in duplicate into six of the tubes containing brain extract and adsorbed bovine plasma. The tubes containing the mixtures are then placed in the water-bath for three minutes before recalcification. Directly before the set time has elapsed the next set of test plasma samples are pipetted out. By this means it is possible to avoid any time wastage due to incubation. It must be emphasized that the test plasma should not be diluted more than 30 minutes before use, and that the bovine

plasma and brain extract should also not be mixed more than 30 minutes before use.

B. Preparation of Dilution Curve.—The result is expressed as a "concentration" from a dilution curve plotted on double logarithmic paper. The preparation of this dilution curve is possibly the most important of the preparatory measures for this test. It has been found that different samples of normal plasma when diluted frequently give curves of different slopes even if the coagulation times of the undiluted plasmas are similar. Therefore all dilution curves used in this test are prepared from pooled normal plasma, the pool usually being a minimum of five normal samples. To maintain constant ionic strength all concentrations of the pooled plasma (except the 100%) have to be diluted twice; it is essential that the dilutions are made as carefully as possible. The following concentrations are usually sufficient:

100% (pooled plasma diluted 1/10 in sol. II).
50% (pooled plasma diluted 1/2 in sol. I then 1/10 in sol. II).
20% (pooled plasma diluted 1/5 in sol. I then 1/10 in sol. II).
10% (pooled plasma diluted 1/10 in sol. I then 1/10 in sol. II).

The above dilution curve should be made to test each new reagent (thromboplastin or bovine plasma). At least four estimations should be made on each concentration, and it is preferable to use two separate pooled samples of normal plasma. The mean coagulation times are plotted against "concentration" on double logarithmic graph paper, when a straight line should be obtained. The graph in routine use in this laboratory is shown in Fig. 2. It is particularly important to have multiple readings for the 10% and 20% dilutions of normal plasma because these show rather wide variations from one sample to another and the clotting times are much affected by small dilution errors.

It will be noted that on the graph the results are expressed simply as "concentration" as a percentage of normal. The method is not designed to measure any one substance, thus it is impossible to use specific terms. For routine reporting some phrase such as "one-stage prothrombin time by Owren's method, 105 seconds normal, 36 seconds 17% of normal" can be used.

C. Effect of Different Types of Adsorbed Bovine Plasma.—In the early stages of this investigation a comparison was made between three different methods of adsorbing the bovine plasma.

(*a*) Seitz filtration, when bovine plasma is passed through two grades of Seitz filter pad, containing 20% and 50% asbestos. The pads were manufactured by the Republic Seitz Filter Corp. of the U.S.A., the appropriate grades being G.P. for the first filtration and S3 for the second.

(*b*) Seitz filtration, as above except that Ford's "sterimats" were used. Grade F.C.B. was used for the first filtration and Grade SB for the second.

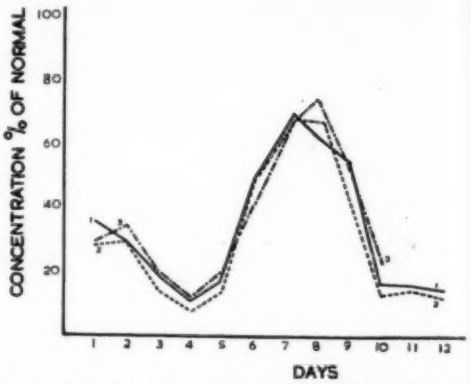

FIG. 1.—Daily estimations on a patient receiving "dindevan" using three different bovine plasma preparations: (1) Adsorption by Seitz filtration, (2) adsorption by Ba_2SO_4, (3) adsorption by Seitz filtration using Ford's "sterimats."

(*c*) Adsorption was achieved with barium sulphate (see above).

Patients being followed with Owren's method had daily estimations made with all three bovine plasma preparations. In all some 100 blood samples were tested, and no significant difference was found between the three preparations (Fig. 1). In this department the third method of adsorption was adopted as being the most suitable. In other departments the filtration method may be found easier if sufficiently large filters are available.

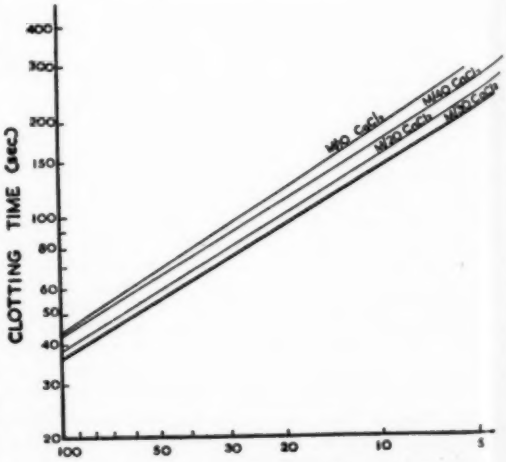

FIG. 2.—The effect of different calcium chloride concentrations on the pooled normal plasma dilution curve. The thick line for M/30 $CaCl_2$ is similar to the curve used routinely.

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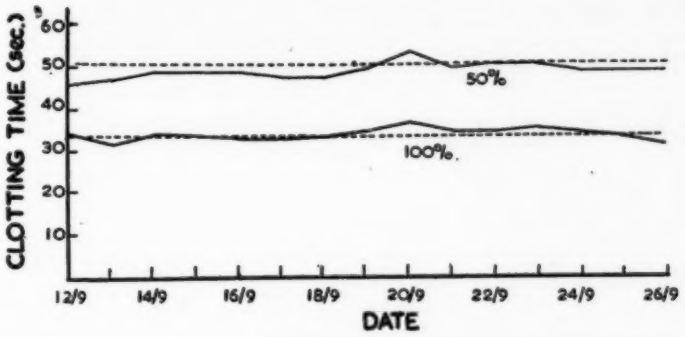

FIG. 3.—Daily estimations on stored pooled normal plasma of the 100% and 50% levels. The dotted lines represent the 100% and 50% levels for the dilution curve in routine use at that time.

D. Effect of $CaCl_2$ Concentration on Dilution Curve.—The optimum $CaCl_2$ concentration is rather critical and may vary slightly for different batches of bovine plasma and brain extract. It is therefore of some importance that the optimum $CaCl_2$ concentration is found for each new reagent. The concentrations used are M/20, M/30, and M/40. Usually the M/30 concentration gives the better times, though occasionally M/20 has been selected. The effect of differing concentrations on the 100% and 50% levels of pooled normal plasma is usually quite small, whereas with the high dilutions of pooled normal plasma the effect will be more marked (Fig. 2).

E. Stability of Reagents over a Prolonged Period.—The bovine plasma and the brain extract appear to be stable over quite a prolonged period. No significant alteration has been detected over a period of three to four months. As it is unusual to prepare enough of these substances to last longer than this, tests over a longer period have not been made.

F. Daily Standardization of Method.—In routine use this method is very nearly self-standardizing from day to day. A steady supply of new patients assures the 100% level and usually there are a number of well stabilized patients, and by observing results on the samples from these patients any gross systematic deviation would be obvious. Also frequent estimations have been made of the 50% and 100% dilutions of stored pooled normal plasma. The results on the pooled samples have remained very constant (Fig. 3). Initially 10% and 20% dilutions were made daily, but this procedure gave rise to a good deal of unnecessary confusion because the clotting times of mixtures containing these high dilutions tended to be rather variable. The patient's plasma is always tested at the 1 in 10 dilution (100%) and the higher dilutions of the dilution curve are determined on multiple samples when random variability is reduced to a minimum. It therefore seemed best to adhere to the procedure used by the originators of the technique and test daily only the 1 in 10 and 1 in 20 dilutions of the normal plasma (100% and 50%).

G. Introduction of New Reagents.—It has been found that when a new batch of bovine plasma or thromboplastin or both is brought into routine use there is very little change in levels. Patients well stabilized should show only minimal alteration on the change-over. In routine practice daily estimations with the old and new reagents have been made for two or three days before the new reagent is adopted. The results given should always be in close agreement. An example of the sort of differences that may be expected in changing from one set of reagents to another is shown in Table I.

TABLE I
EFFECT OF DIFFERENT REAGENTS

Plasma	Concentration (%) of Normal	
	Thromboplastin I Bovine Plasma I	Thromboplastin II Bovine Plasma II
James	23	21
Mores	22	18·5
Anderson	29	27
Morwood	23	21
Collins	30	27
Osborne	34	28
Howard	88	30
Finch	10	9·2

The Clinical Application of the Method

The test is carried out daily until a stable level is reached and thereafter estimations are made on alternate days while the patient remains in hospital. If long-term treatment is given to out-patients the test is done at weekly or fortnightly intervals.

To establish the correct stabilizing dose there is no doubt that graphical representation of the results is helpful. General trends in the levels are much more obvious if seen graphically. A comparison between the results of Owren's method and the unmodified one-stage test is shown in Figs. 4 and 5. The anticoagulant dosage for both of these patients was calculated from the results of the unmodified one-stage test and Owren's method was at the time used experimentally. It is clear from a first impression that the two methods give roughly corresponding results. It is also clear that

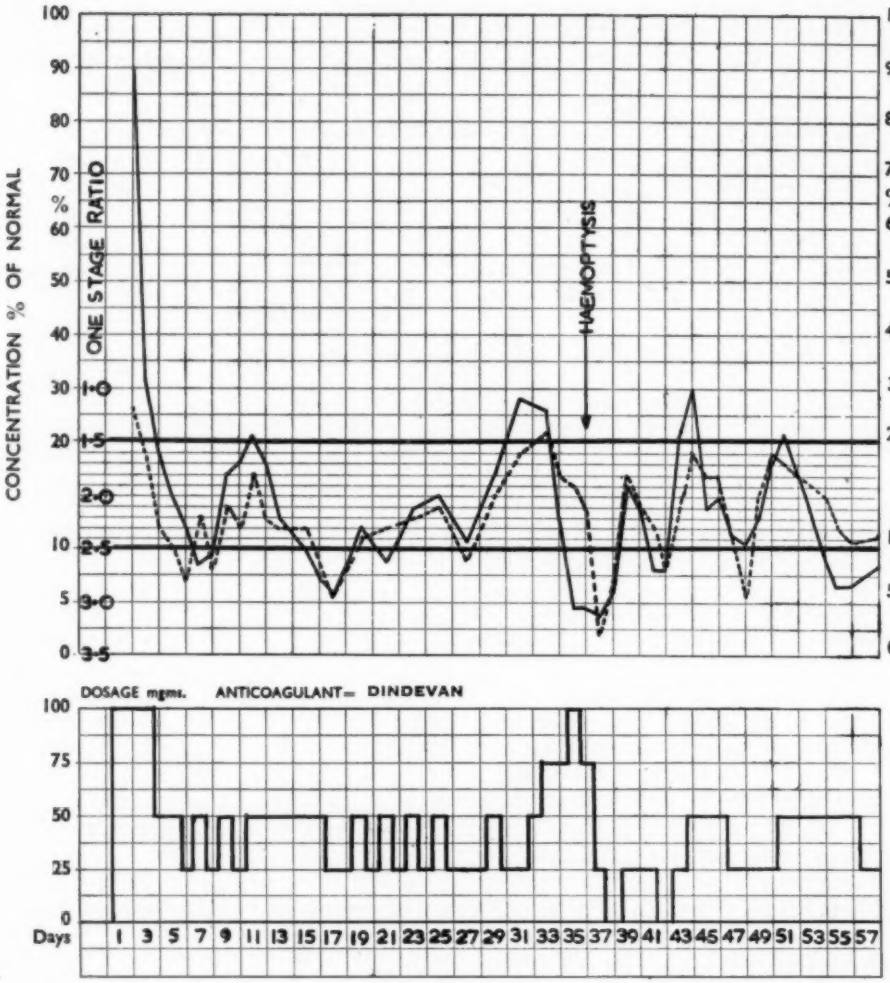
CONCENTRATION % OF NORMAL
ONE STAGE RATIO %
1·0
1·5
2·0
2·5
3·0
3·5
HAEMOPTYSIS
DOSAGE mgms. ANTICOAGULANT= DINDEVAN
Days
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57

FIG. 4

FIGS. 4 and 5.—A comparison between Owren's method and the unmodified one-stage method on two patients receiving "dindevan." The unmodified one-stage method is expressed as a ratio = $\frac{\text{Patient's clotting time}}{\text{Normal clotting time}}$. The therapeutic range for both methods lies between the thick parallel lines. The charts shown are used in this hospital for graphing anticoagulant therapy data. Dotted line indicates one-stage ratio, the solid line Owren's method.

according to the results of Owren's method neither of the patients was ever established on an exactly balancing dose.

Detailed examination indicates the main sources of difficulty. In Fig. 4 it can be seen that from the eleventh to the sixteenth day of treatment the patient received 50 mg. of "dindevan" daily. The results of Owren's method show a slow but unmistakable downward trend. This finally becomes apparent on the unmodified method on the seventeenth day when the dose was reduced. The dose was thereafter very nearly stabilized at about 37.5 mg. daily. A single random low reading for the one-stage method led to reduction and then to a

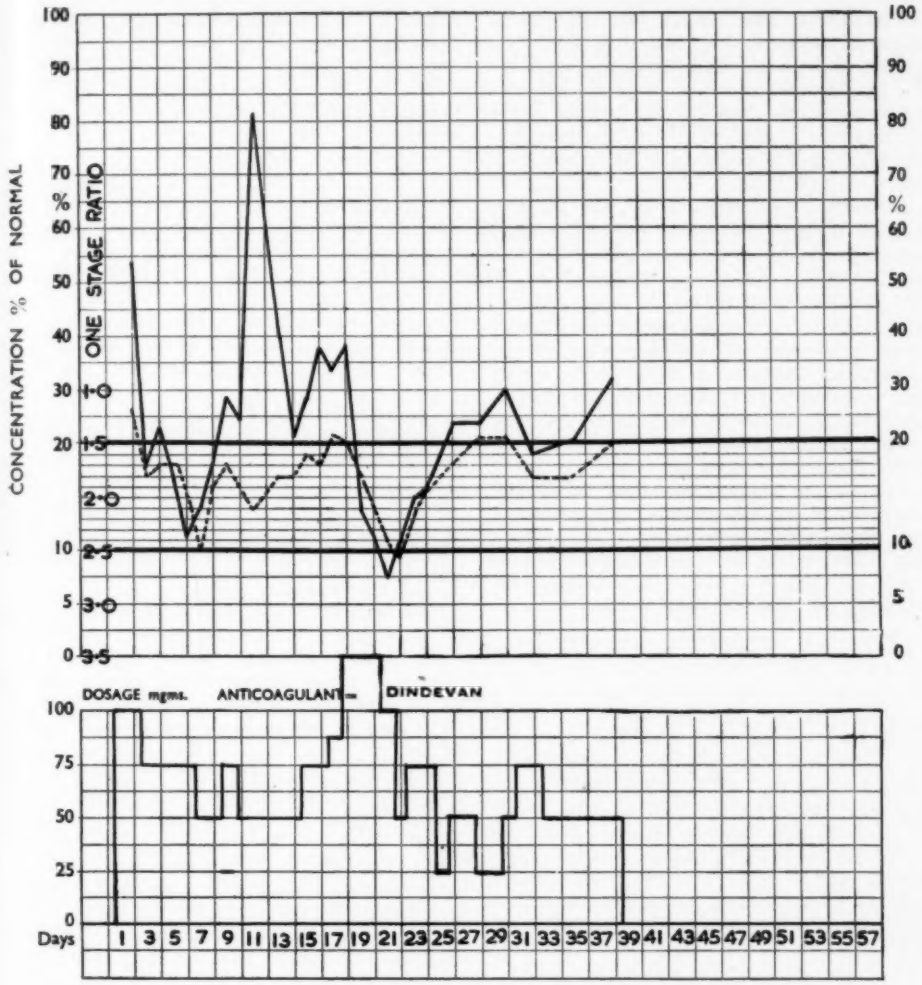

FIG. 5

very large and unjustified increase of dosage, the results of which are well reflected by the curve for Owren's method.

In Fig. 5 it is also clear that the failure to stabilize the dosage was mainly due to the very indefinite day-to-day trend of prothrombin times using the unmodified method. The balancing dose for this patient was probably of the order of 75 mg. daily.

The results obtained by any one-stage method reflect the dosage on the previous day with the addition of any drug cumulated from previous excessive doses. To reach a stabilizing dose it is therefore essential to apply an increasingly fine adjustment to the dose. Thus if 100 mg. doses are given on the first three days (Fig. 4) the effects of this treatment will be apparent in the results of testing on the fourth, fifth, and even sixth days. During this period an approximate dose is selected and, according to the general trend during the next four or five days, the dose may be altered by a small adjustment. Large dose alterations are very

seldom necessary and always lead to serious imbalance in treatment (Figs. 4 and 5).

Detailed study of records such as these emphasizes the need for small dose adjustments. This is now possible since the introduction of the 10 mg. tablets of "dindevan." Using combinations of the 10 and 50 mg. tablets the dose may be adjusted to within 5 mg.

In the initial investigation of the method, patients receiving anticoagulant drugs had daily estimations made by both Quick's and Owren's method. About 1,500 blood samples from 63 patients were included in the survey. Almost invariably Owren's method showed the different trends in levels and hence dosage much sooner than did Quick's method. Also the day-to-day random variation was much smaller with Owren's method, making the trends much more obvious. The large random variation of Quick's method tended to mask rising or falling levels.

Owren's method has now been used for all anticoagulant work in this hospital group for 20 months. During this time nearly 12,000 estimations have been made, and the method has proved eminently satisfactory. The small random variation makes the method very suitable for outpatients receiving long-term therapy. It is also an advantage in that blood specimens can be sent by post with little effect on the result, making it possible for general practitioners to collect the samples in districts at some distance from the hospital.

Summary

A description is given of the P and P (prothrombin and proconvertin) method of Owren as used routinely in this department for the control of anticoagulant therapy. The method has been found to be more sensitive and subject to less random variation than Quick's one-stage method and to be particularly suitable for the control of long-term anticoagulant therapy.

I am grateful to Dr. Rosemary Biggs for her advice and encouragement during all stages of the preparation of this paper.

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THE ISOLATION OF CHROMOGENIC MYCOBACTERIA FROM LARYNGEAL CULTURES

BY

MIKLÓS LÁNYI

From the University Medical School, Budapest

(RECEIVED FOR PUBLICATION MARCH 22, 1957)

It is known that the cultural characteristics of strains of *Mycobacterium tuberculosis* isolated from tuberculous secretions may be changed under the influence of anti-tuberculosis drugs. It has been suggested that this is due to "biological disintegration" of the bacteria (Lányi and Eidus, 1954). The process is usually a gradual one. The present report is concerned with the isolation from laryngeal swabs of a number of atypical chromogenic strains of mycobacteria presumed to be disintegrated tubercle bacilli.

Source of Strains

In the routine culture for tubercle bacilli of 2,620 laryngeal swabs, atypical, chromogenic strains of mycobacteria were isolated on 16 occasions. The strains came from 16 patients, all suffering from pulmonary tuberculosis, and in 13 typical tubercle bacilli had been repeatedly isolated from previous swabs. Three of the patients appeared to have regressive infiltrative lesions, and the others, including nine with bilateral haematogenous apicosclerosis, suffered from chronic fibrosis. Thus the pathological condition was that seen in patients with a good immunity, and the clinical condition of all was satisfactory. The patients had been treated by chemotherapy, and the average dose received was 24.4 g. isoniazid, 827 g. P.A.S., and 32.8 g. streptomycin.

Characteristics of Atypical Mycobacteria

The strains caused a brownish discoloration on liquid lyophilized bovine plasma medium (Kováts, Eidus, and Lányi, 1955) and their colonies were easily shaken apart and settled down slowly. On Löwenstein medium the strains produced smooth, dysgonic colonies, which formed yellow, orange-red or brownish-red pigments. Pigment production increased in daylight. Cultures on glycerine-free solid medium grew more abundantly. The primary culture period was 24–28 days at an optimal temperature of 37° C. They failed to grow on simple agar or broth media. All strains were resistant to 10 μg. per ml. isoniazid.

The organisms were Gram-positive, acid-fast bacilli, showing many granules and placed in cord formation, similar to typical tubercle bacilli. They gave a positive neutral red test but were catalase negative.

Subcutaneous inoculation of guinea-pigs with 0.5 mg. wet weight caused a slight local reaction only, without spread, but the animals became tuberculin positive.

Discussion

Chromogenic acid-fast bacilli have been isolated from human sources by many authors. Some of these bacilli appeared to be quite different from tubercle bacilli and caused only benign local lesions (Linell and Nordén, 1954; Wells, Agius, and Smith, 1955). In other cases the strains were isolated from patients with atypical pulmonary lesions (Buhler and Pollak, 1953; Ostry, Kubin, *et al.*, 1956; Wood, Buhler, and Pollak, 1956; Young, 1955). The pathogenic significance of these strains in man has already been discussed (Tarshis, 1956; Tarshis, Weed, Parker, and Dunham, 1955; Tarshis and Frisch, 1952; Timpe and Runyon, 1954), and it appears that those strains which are resistant to isoniazid are not pathogenic (Middlebrook, Cohn, and Oestreicher, 1955). Wilson and Morton (1956) described 46 atypical non-pathogenic acid-fast bacilli, but only nine were chromogenic and not all the patients from whom the strains were isolated had received chemotherapy.

The chromogenic strains recorded in this paper differ in many respects from saprophytic mycobacteria. Moreover, laryngeal swabs are not usually contaminated with saprophytic acid-fast bacilli. It is, therefore, presumed that these strains, isolated from patients with a good immunity after chemotherapy, represent dis-

integrated tubercle bacilli. It is possible that a state of equilibrium is reached between these disintegrated mycobacteria and the host.

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A METHOD FOR TYPING *SHIGELLA SONNEI*, USING COLICINE PRODUCTION AS A MARKER

BY

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(RECEIVED FOR PUBLICATION APRIL 25, 1957)

Infection with *Shigella sonnei* is the most common identifiable cause of diarrhoea in this country at present, and the absence of a satisfactory method of typing the organism has greatly hampered the study of its mode of spread. Bojlén (1934), studying the biochemical reactions of 1,786 Sonne cultures, isolated from 741 patients in Denmark, described four biochemical types based upon the fermentation of maltose and xylose. However, when these cultures were examined at a later date only xylose fermentation was found to be stable. Although other workers (Cruickshank and Swyer, 1940 ; Tee, 1955) found xylose fermentation useful for tracing Sonne infections, the method has only a limited value in this country ; for example, Tee (1955) examined cultures from 812 patients and found that only 44 (5.4%) fermented xylose.

Phage-typing of *S. sonnei* was first reported by Hammarström (1947) in Sweden, and appears to have proved useful to some workers (Ludford, 1953 ; Szturm-Rubinsten and Thibault, 1956). In this country the method was used by Mayr-Harting (1952) and by Tee (1955). The latter, who tested 829 Sonne cultures with 10 unadapted phages, concluded: "This method of phage-typing, because of evidence of type instability and because the majority of strains belong to the same phage-type, has little practical value as a routine procedure in the control of Sonne dysentery in this country."

Davies (1954) described a simple disc technique for testing Sonne cultures for sulphonamide sensitivity. Strains isolated from family or institutional outbreaks were usually either all sensitive or all resistant ; in outbreaks where mixed infections occurred there was evidence of multiple sources of infection. Occasionally, exacting strains were found, which required for normal growth the addition of one or more amino-acids to the synthetic medium used for testing the sensitivity to sulphonamides by this method. The dependence upon one or more amino-acids may be used as a "marker" for tracing infection with exacting strains.

None of these procedures has proved entirely satisfactory because no procedure has divided the species into a sufficient number of commonly encountered types.

Fredericq (1946a) tested the sensitivity of a number of non-lactose-fermenting intestinal bacteria, including Salmonellae and Shigellae, to antibacterial substances produced by two strains of *Escherichia coli* and one aerobic spore-bearing bacillus, and showed that they could be divided into groups by means of their pattern of susceptibility to these substances. He suggested that it might be possible to establish for each type of organism a characteristic "antibiotic sensitivity formula" analogous to the antigenic formula already established for members of the Salmonella group. This work led us to investigate the sensitivity of Sonne strains to colicines to see if this property could be used as a basis for a typing method.

Materials and Methods

Media.—The following were used :

Infusion Broth.—A beef infusion to which 1% peptone (Evans) and 0.5% sodium chloride were added, and the *p*H adjusted to 7.4–7.6 (Mackie and McCartney, 1948).

Nutrient Agar.—One per cent. New Zealand agar (Davis) was incorporated in infusion broth.

Lab-Lemco Agar.—One per cent. New Zealand agar (Davis) was incorporated in Lab-Lemco infusion broth (Mackie and McCartney, 1948), and the final *p*H adjusted to 7.4–7.6.

Blood Agar.—Ten per cent. horse blood was incorporated in nutrient agar or Lab-Lemco agar.

Dorset's egg medium was prepared according to the formula of Mackie and McCartney (1948).

MacConkey agar was prepared according to the formula of the Ministry of Health (1956).

Incubation.—Incubation was aerobic at 35° C. to 36° C.

Identification of Cultures.—All strains of *S. sonnei* were plated out on to MacConkey's agar and their identity confirmed by colonial morphology and slide agglutination with S-R Sonne antiserum (Standards Laboratory).

Maintenance of Cultures.—Cultures were stored on Dorset's egg medium in screw-capped bottles at room temperature in the dark.

Sulphonamide Sensitivity Tests.—The method described by Davies (1954) was used.

Testing for Colicine Production.—A simple method was devised by one of us for testing *Esch. coli* O55 B5 cultures for colicine production and for sensitivity to colicines (Shannon, 1957). This method, a modification of one used by Fredericq, Thibault, and Gratia (1946), was found to be suitable also for *S. sonnei*. The culture to be tested for colicine activity (the active strain) was inoculated heavily from MacConkey agar across a dried blood-agar plate to give a confluent streak of growth (the primary streak); the purpose of the horse blood was to act as a source of catalase to eliminate inhibition due to hydrogen peroxide formation (Gardner, 1950). After a suitable period of incubation, the active strain was killed by exposure to chloroform. For this purpose a disc of filter paper was placed in the lid of a Petri dish and saturated with chloroform, the portion containing the medium was replaced and the plate left inverted on the bench. After one hour's exposure to chloroform vapour, the growth was scraped to one end with the edge of a clean slide and then removed together with a small portion of agar. More chloroform was added to keep the filter paper moist and the plate was left closed for a further hour on the bench. The filter paper was then removed, and the plate exposed to the air on the bench for three hours with the lid open and the surface of the medium face down. Overnight broth cultures of organisms to be tested for sensitivity to colicines (the passive or indicator strains) were then inoculated across the plate at right angles to the position formerly occupied by the primary streak. The passive strains were inoculated with a 3–4 mm. loop so as to give a regular confluent growth across the plate. When inoculating a large number of plates, it was found unnecessary to flame the loop between each plate; it was sufficient to flame the loop before it was introduced into a broth culture of a different organism. The plates were incubated overnight and examined the next day.

If no colicine active against any of the passive strains was produced, all grew across the plate as parallel confluent streaks. If such a colicine was produced, areas of inhibition of one or more of the passive strains were seen, distributed symmetrically on either side of the original line of inoculation of the active strain.

The results were read as follows:

+++ = Inhibition zone >4 mm.
++ = " " 2–4 mm.
+ = " " <2 mm.
± = Thinning only
− = No inhibition
R = Presence of numerous resistant colonies in zone of inhibition
D = A characteristic form of inhibition consisting of a central area of growth with a zone of inhibition on either side.

Thus, +++R indicates a wide zone of inhibition with numerous resistant colonies, and ++D a central plateau of growth with a moderate zone of inhibition on either side. In reading the results, the width of inhibition zones was not measured accurately but estimated approximately.

This technique of testing colicine production was used in all the experiments, although the incubation period of the primary streak and the composition of the blood-agar medium were varied.

Preliminary Investigation

Lab-Lemco blood-agar plates were used in these tests; the primary streak (active strain) was incubated for 48 hours before exposure to chloroform vapour.

Sensitivity of S. sonnei to Colicines.—It seemed possible that *S. sonnei* cultures might be divided into groups by means of their susceptibility to colicines produced by other organisms.

Therefore, 50 *Esch. coli* cultures isolated from routine faecal specimens were tested for colicine activity against 46 Sonne strains that had been sent from various parts of England. Fifteen of the *Esch. coli* cultures produced colicines; some inhibited all 46 Sonne cultures, while others inhibited only a few. The 15 active *Esch. coli* cultures were then tested against a number of epidemiologically related Sonne strains. Different sensitivity patterns were often shown by strains that were considered identical from epidemiological evidence.

Next, each of the 46 Sonne strains were tested for colicine activity against itself and against the other 45 Sonne strains. The results confirmed the work of Fredericq (1948a), who found that many Sonne cultures were active against other Sonne cultures, although a particular strain was never sensitive to its own colicine. The sensitivity of groups of epidemiologically related cultures to the colicines produced by a number of active strains was then tested. Once again, strains that might have been expected on epidemiological grounds to be identical often showed different sensitivity patterns.

It was unlikely, therefore, that the sensitivity patterns of Sonne strains to colicines could be

used as a basis for a typing method, since related strains often differed in their sensitivity to colicines produced by cultures of *Esch. coli* or *S. sonnei*.

Production of Colicines by Shigella sonnei.—The preceding experiments had shown that *S. sonnei* cultures frequently produced colicines active against other strains of *S. sonnei*. We therefore investigated the production of colicines by Sonne strains to find out whether this property could be used as a basis for a typing method.

A number of related Sonne strains were tested for the production of colicines active against a series of passive (indicator) strains selected from the 46 Sonne cultures used in the previous experiments. The results were encouraging, since cultures considered identical from epidemiological evidence produced colicines active against the same indicator strains. Moreover, consistent results were obtained when the tests were repeated.

The Inhibition Patterns Produced by Sonne Cultures on Selected Indicator Strains

Lab-Lemco blood-agar medium was used; the period of incubation of the primary streak was extended to four days, as certain Sonne strains (type 2) produced weak and variable inhibition zones after two days' incubation. From the 46 Sonne cultures used in the previous experiments, six indicator strains which divided certain Sonne cultures into types by means of clear-cut inhibition zones were selected; indicator strains giving variable results, or inhibition zones that were weak or overgrown with resistant variants, were rejected.

One hundred Sonne cultures, mostly isolated in the Manchester area, were tested for colicine activity against the six indicator strains. Over a half of the 100 Sonne cultures could be classified into one of three types according to the inhibition patterns produced upon the indicator organisms. The inhibition patterns of the three types are shown in Table I. The commonest type was type 1, type 2 was found less frequently, and type 3 cultures were isolated on three occasions only. The remaining cultures were classed as untypable; these included some strains that inhibited none of the indicator strains and others giving inhibition patterns different from those shown in Table I.

TABLE I
INHIBITION PATTERNS OF TYPABLE STRAINS OF *S. SONNEI* ON SIX INDICATOR STRAINS

Indicator Strains and Lab. No.	Provisional Colicine Type		
	1	2	3
S. sonnei 2	+++	−	+++
" 56	+++	+++	+++
" 17	+++	+++	+++
" 38	−	−	+++
" 2M	−	−	−
" 10	−	−	+++

Stability of Indicator Strains.—Indicator strains were maintained on Dorset's egg medium with infrequent subculture, and stored at room temperature in the dark. With one exception—*S. sonnei* 10—the sensitivity of the indicator strains remained stable over a period of several months. *S. sonnei* 10 was later discarded since its sensitivity to type 1 cultures varied slightly in successive tests. The stability of these indicator strains confirms the work of Fredericq (1948b), who found that the sensitivity or resistance of a culture to a particular colicine was usually stable; the sensitivity of certain strains that were tested by him many times over a period of four years remained the same.

Stability of Colicine Production.—Fredericq (1948b) showed that colicine production is a stable property. It is usually unaffected by changes in morphology, in biochemical reactions, or in antigenic structure—such as S-R variation (Fredericq, 1946b). Our experiments confirmed these findings; Sonne cultures produced similar inhibition patterns when tests were repeated many times over a period of several months; colicine production was unaffected by changes in colonial morphology.

Consistency of Typing Results.—Reproducible results were obtained when cultures were tested over a period of several months. A number of different colonies of *S. sonnei* selected from the primary cultures of faeces on deoxycholate-citrate agar were tested for colicine production. An average of six colonies from primary cultures of nine type 1 strains were examined, and all gave similar inhibition patterns. Repeated isolations from the same patient were tested on a number of occasions, with identical results. In view of these encouraging results, attempts were made to type a further series of Sonne strains, using a similar, but slightly modified, technique.

Typing of 537 Sonne Strains by Means of Colicine Production

Layered blood-agar plates made with nutrient agar were used instead of Lab-Lemco blood-agar. The period of incubation of the primary streak was reduced from four to three days, since satisfactory inhibition zones were obtained after three days' incubation on the nutrient agar medium.

The source of the Sonne cultures examined was as follows:

Public Health Laboratory, Manchester	314	strains
Public Health Laboratory, County Hall, London ...	142	,,
Public Health Laboratory, Wakefield	50	,,
Other laboratories	31	,,

Indicator Strains.—The number of indicator organisms was increased to 14. These comprised five of the cultures—*S. sonnei* 2, 56, 17, 2M and 38—that had been used previously (Table I), one culture of *S. schmitzi* N.C.T.C. No. 8218—and a further eight strains obtained by selecting resistant variants of some of the five Sonne cultures. Fredericq (1947) classified colicines produced by *Esch. coli* and *S. sonnei* by their action upon a carefully selected series of resistant variants. He showed also that resistant variants usually retain their resistance indefinitely; for instance, some resistant mutants retained their resistance after 300 subcultures, although others occasionally recovered some of their original sensitivity (Fredericq, 1948b). By selecting resistant colonies from the inhibition zones of some of the indicator strains, and using these resistant variants as indicator organisms, we were able to provide new indicator strains to check the identity of known colicine types and to distinguish new colicine types. Once again, we selected only those cultures that gave clear-cut reproducible reactions of value in distinguishing the different types. The source from which the resistant variants were derived is shown in Table II. In

TABLE II

SOURCE OF RESISTANT VARIANTS USED AS INDICATOR STRAINS

Indicator Strains and Lab. No.	Indicator Strain from which Resistant Colony was Selected	Provisional Colicine Type of Active Strain to which Selected Colony was Resistant
S. sonnei 56 56	56	2
,, 56/98	56	2
,, R1	2	1b
,, R6	56	1a
,, 2/7	2	1a
,, 2/64	2	Untypable
,, 2/15	2	1a
,, R5	2/7	5

addition, each of the Sonne cultures used as an indicator organism was itself tested for colicine production. The results of typing these organisms are shown in Table III. As the work of Fredericq (1948c) would lead one to expect, each of the resistant variants produced the same inhibition patterns as the parent strain. The sensitivity or resistance to colicines of the 14 indicator organisms remained unaltered over a period of 12 months.

TABLE III

PROVISIONAL COLICINE TYPE OF SONNE CULTURES USED AS INDICATOR STRAINS

Indicator Strains and Lab. No.	Provisional Colicine Type
S. sonnei 2, R1, 2/7, 2/64, 2/15, R5	2
,, 56, 56/56, 56/98, R6	Untypable
,, 17	3
,, 2M	1b
,, 38	

Colicine Types.—By means of the inhibition patterns produced upon the 14 indicator organisms, two-thirds of the Sonne cultures could be divided into seven colicine types (Table IV); each of the

TABLE IV

INHIBITION PATTERNS OF TYPABLE STRAINS OF *S. SONNEI* ON 14 INDICATOR STRAINS

Indicator Strains and Lab. No.	Provisional Colicine Type						
	1a	1b	2	3	4	5	6
S. sonnei 2 ..	+ + +	+ + +	–	+ + +	+ + +	+ + +	–
,, 56 ..	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
,, 17 ..	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	–
,, 2M ..	–	–	–	–	–	–	–
,, 38 ..	–	–	–	+ + +	or ± + + +	or ± + + +	–
,, 56 56	+ + +	+ + +	–	+ + +	–	+ + +	+ + +
,, 56 98	+ + +	+ + +	–	+ + +	–	+ + +	+ + +
,, R1 ..	–	–	–	+ + +	+ + +	+ + +	–
,, R6 ..	–	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
S. schmitzi N.C.T.C. No. 8218	+ + + or + +	+ + + or + +	–	+ + +	–	+ + + or + +	–
S. sonnei 2/7 ..	–	–	–	+ + +	+ + +	+ + +	–
,, 2/64 ..	–	–	–	+ + +	+ + +	+ + +	–
,, 2/15 ..	–	–	–	+ + +	+ + +	+ + +	–
,, R5 ..	–	–	–	+ + +	–	–	–

seven types gave characteristic reactions which were reproduced when tests were repeated. Three new types (4, 5, and 6) were added to the three previously distinguished, and type 1 was subdivided into types 1a and 1b by the reaction of the indicator organism R6. The inhibition pattern

TABLE V

TYPING RESULTS OF 537 STRAINS OF *S. SONNEI*

	Sulphonamide Sensitivity	Provisional Colicine Type							Total No. of Strains
		1a	1b	2	3	4	5	6	Untypable	
No. of strains isolated from family outbreaks	Sensitive	—	—	41	—	6	—	—	29	76
	Resistant	10	30	45	—	4	9	6	14	118
	Exacting	—	—	10	—	—	—	—	1	11
No. of strains isolated from institutional outbreaks	Sensitive	—	—	31	—	11	—	—	63	105
	Resistant	21	44	20	2	3	4	—	15	109
	Exacting	—	—	9	—	—	—	—	—	9
No. of other strains	Sensitive	—	—	12	—	—	—	2	33	47
	Resistant	3	10	8	1	3	9	6	14	54
	Exacting	—	—	7	—	—	—	—	1	8
Total No. of strains ..		34	84	183	3	27	22	14	170	537

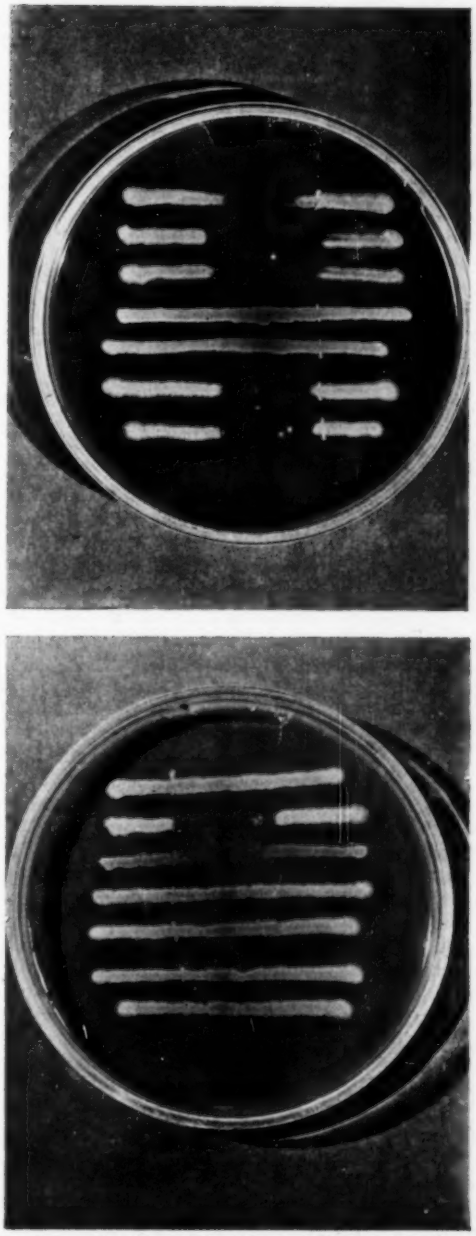

FIG. 1.—The inhibition pattern produced by a strain of *S. sonnei* of colicine type 1b on the 14 indicator strains.

produced by a type 1b strain is shown in Fig. 1. The remaining one-third of the Sonne cultures, classified as untypable, included strains with no action on any of the indicator organisms, and other strains giving variable results or inhibition patterns different from those shown in Table IV. The production of colicines by the seven colicine types appears to be stable. A number of strains of each type was re-examined after six months' storage on Dorset's egg medium, and gave similar inhibition patterns to when they were first tested. There is some evidence, however, that cultures stored for longer periods may show weaker reactions. As some of the indicator strains give identical reactions, it would be possible to distinguish the seven types with fewer than 14 indicator organisms. At present, however, we are continuing to use the full series of indicator strains, to detect possible variations in sensitivity of the indicator organisms and to increase the chance of recognizing new types. Two blood-agar plates are therefore needed to type each culture, with seven indicator strains on each plate.

Results.—From March to September, 1956, 537 Sonne cultures were tested for colicine production and for sulphonamide sensitivity; 367 were classified into one of the seven colicine types and 170 were untypable (Table V). Approximately one-third of the cultures belonged to type 2, one-third were untypable, and the remaining one-third comprised members of the other six types.

The relationship of the colicine types to sulphon-

amide sensitivity is also shown in Table V. It is of interest that all strains of types 1a, 1b, 3, and 5 were resistant to sulphonamide.

One hundred and thirty-four outbreaks were investigated. In this context we define an outbreak as two or more isolations of *S. sonnei* within a period of four weeks from different persons in a family or institution. The colicine type was uniform in 97 outbreaks (306 out of 456 strains) and mixed in five (18 out of 456 strains); in 32 outbreaks the strains were untypable (132 out of 456 strains). The sulphonamide sensitivity was uniform in 127 outbreaks (431 out of 456 strains) and mixed in seven (25 out of 456 strains). The distribution of colicine types and sulphonamide sensitivity in the outbreaks with uniform colicine type and sulphonamide sensitivity is shown in Table VI. We have also included in this table outbreaks

TABLE VI

DISTRIBUTION OF COLICINE TYPE AND SULPHONAMIDE SENSITIVITY IN OUTBREAKS WITH *S. SONNEI* STRAINS OF UNIFORM COLICINE TYPE AND SULPHONAMIDE SENSITIVITY*

	Sulphonamide Sensitivity	Provisional Colicine Type								Total no. Outbreaks
		1a	1b	2	3	4	5	6	Untypable	
No. of outbreaks in families	Sensitive	—	—	17	—	3	—	—	9	29
	Resistant	4	9	18	—	2	4	2	5	44
	Exacting	—	—	3	—	—	—	—	—	3
No. of outbreaks in institutions	Sensitive	—	—	9	—	4	—	—	13	26
	Resistant	4	8	5	1	1	1	—	2	22
	Exacting	—	—	2	—	—	—	—	—	2
Total no. of outbreaks		8	17	54	1	10	5	2	29	126

*Outbreaks with untypable strains of uniform sulphonamide sensitivity are also included.

with untypable strains which were of uniform sulphonamide sensitivity. Type 2 strains were found in about half of the outbreaks, untypable strains in a quarter, and other colicine types in the remainder. From the number of uniform outbreaks with type 1a, 1b, 2, 4, and 5 strains, it seems probable that these correspond to genuine epidemiological types; the number of infections with type 3 and type 6 strains is, as yet, too few to assess their value as epidemiological types.

The distribution of colicine types and sulphonamide sensitivity in the mixed outbreaks is shown in Table VII. These comprised four with mixed colicine type and mixed sulphonamide sensitivity, and one with mixed colicine type and uniform sulphonamide sensitivity; in three outbreaks the sulphonamide sensitivity was mixed but the strains were untypable. It seems likely that outbreaks with mixed colicine types represent true mixed infections, rather than instability of colicine types, as they were few and usually associated with a difference in sulphonamide sensitivity.

TABLE VII

DISTRIBUTION OF COLICINE TYPE AND SULPHONAMIDE SENSITIVITY IN "MIXED" OUTBREAKS

Outbreak No.	No. of Strains Isolated	No. of Strains	Sulphonamide Sensitivity	Provisional Colicine Type
Family 1.. ..	2	1	Sensitive	Untypable
		1	Exacting	,,
,, 2.. ..	3	2	Resistant	1a
		1	,,	5
,, 3.. ..	2	1	,,	Untypable
		1	Sensitive	,,
,, 4.. ..	3	2	Resistant	1b
		1	Exacting	2
Institutional 1 ..	4	2	Resistant	1a
		2	Sensitive	2
,, 2 ..	5	4	Resistant	1b
		1	Sensitive	2
,, 3 ..	6	5	Resistant	Untypable
		1	Sensitive	,,
,, 4 ..	3	2	,,	,,
		1	Resistant	2

Discussion

The distribution of strains amongst the different colicine types compares favourably with phage-typing methods. Tee (1955), who examined 829 Sonne cultures by a phage-typing method, found that 74% belonged to one phage type. About two-thirds of the cultures tested could be typed, and the commonest type, type 2, constituted only 29% of all cultures tested.

Colicine typing gave reliable epidemiological information. In 97 of the 102 outbreaks from which typable strains were isolated the colicine type was uniform, and the results were in agreement with the epidemiological evidence. In four of the five outbreaks from which strains of different colicine types were isolated there was also a difference in sulphonamide sensitivity. A small number of mixed outbreaks might be expected with such a common infection as Sonne dysentery.

At present the main disadvantage of the method is the large number of untypable strains, in this series approximately a third of all cultures examined. It may prove possible in the future to reduce this number. Of the 170 strains that were untypable, 126 gave consistently negative results against the 14 indicator strains. If further work confirms that these 126 strains are completely inert, and if they can be clearly distinguished from other untypable strains, some of which produce very weak reactions, it may be possible to classify the inert strains into one group.

Further work is necessary to standardize the conditions of the test. The best results have been obtained by incubating the primary streak for three

days on blood-agar medium prepared with infusion broth. The activity of type 6 strains, in particular, is affected by small changes in the composition of the medium. The optimum temperature of incubation appears to be 35° C.–36° C. Variable results are obtained at lower temperatures, and at higher temperatures the inhibition zones are less clear cut.

It is possible that the detection of colicine production may form the basis of typing systems for other organisms. One of us has had success with a similar technique for typing *Esch. coli* O55 B5.

Summary

(1) The sensitivity of *S. sonnei* to colicines was investigated. It seemed unlikely that colicine sensitivity could be used as a basis for a typing method since the sensitivity of epidemiologically related strains often differed.

(2) A method of typing strains of *S. sonnei* by means of colicine production was described. Strains were divided into types by the inhibition patterns produced upon a series of selected indicator organisms. Related strains produced colicines active against the same indicator organism.

(3) Of 537 cultures of *S. sonnei* examined by this method, 367 were classified into one of seven colicine types and 170 were untypable. The cultures were also tested for sensitivity to sulphonamides.

(4) One hundred and thirty-four outbreaks were investigated. The colicine type was uniform in 97 and mixed in five; in 32 outbreaks the strains were untypable. The sulphonamide sensitivity was uniform in 127 outbreaks and mixed in seven. The outbreaks with mixed colicine type probably represent true mixed outbreaks rather than examples of instability of colicine types.

(5) The method is technically simple, and gives reproducible results. Its value would be enhanced if it proves possible to reduce the number of untypable strains.

We wish to thank Dr. M. T. Parker for encouraging us to undertake this investigation and for much helpful advice. We are also grateful to Dr. A. J. H. Tomlinson and Dr. L. A. Little for cultures and for their advice and help, and to Miss J. Perry for the photographs.

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THE RESPONSE OF URINARY 17-HYDROXY-CORTICOIDS TO CORTICOTROPHIN ZINC AS A TEST OF ADRENAL CORTICAL FUNCTION

BY

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An increased interest in the study of the adrenal cortex, together with the ready availability of corticotrophin (A.C.T.H.), have, in recent years, led to the development of improved methods for the assessment of adrenal cortical function. The first of such methods was devised by Thorn, Forsham, Prunty, and Hills (1948) and depended upon the fall in blood eosinophils after injection of a single dose of corticotrophin. It was subsequently realized, however, that this test was by no means specific, since a fall in eosinophils could be produced by the injection of adrenaline, for example, without any effect on the adrenal steroids (Nelson, Sandberg, Palmer, and Glenn, 1952). Moreover, there is a considerable spontaneous variation in the eosinophil count and results may be difficult to interpret when the initial count is low.

The urinary 17-ketosteroid excretion after corticotrophin provides a more valuable indication of adrenal cortical function and many of the current tests rely on this estimation. In males, however, the 17-ketosteroids are only partly derived from the adrenal cortex, and sometimes a clearly defined rise after corticotrophin is not obtained even in normal individuals. Jenkins, Forsham, Laidlaw, Reddy, and Thorn (1955) find that 14% of normal controls showed a rise of less than 2 mg. in 24 hours.

Recently, relatively simple methods have been devised for the estimation of urinary 17-hydroxycorticoids, and it seems that these methods provide a more direct measure of adrenal cortical function since the 17-hydroxycorticoids represent that fraction of the adrenal steroids known as the glucocorticoids, of which hydrocortisone and its urinary metabolites are the most important. It is now recognized that for an adequate assessment of its function a prolonged stimulation of the adrenal cortex is necessary. Jenkins *et al.* (1955) describe the use of an eight-hour intravenous infusion of corticotrophin, but this is inconvenient, and occasional anaphylactic reactions have been reported. The same authors find that corticotrophin gel injected intramuscularly is equally effective in producing a rise in 17-hydroxycorticoids. Recently, a very effective aqueous suspension of corticotrophin and zinc phosphate has been introduced, which is more easily injected than the gel preparation. Greene and Vaughan-Morgan (1954), den Oudsten, van Leeuwen, and Coers (1954), and Ferriman, Anderson, and Turner (1954) have shown that corticotrophin-zinc phosphate has a prolonged and powerful stimulatory effect on the adrenal cortex, as reflected by the eosinophil count and 17-ketosteroid excretion. The purpose of the present report is to describe a test of adrenal cortical function based upon the estimation of urinary 17-hydroxycorticoids before and after the administration of corticotrophin zinc.

Methods

Twenty-four-hour urine collections were started at 9 a.m. The first day was used as a control, and on the second day 40 units of corticotrophin zinc was injected intramuscularly at 9 a.m. and 9 p.m. In most cases urine collection was continued for the third day.

Urinary 17-hydroxycorticoids were estimated by the method of Reddy, Jenkins, and Thorn (1952) as modified by Reddy (1954) and involved the extraction of urine with butanol followed by the colour reaction of Porter and Silber (1950). This method has the merit of being relatively simple to perform and rapid enough for estimations to be completed within a few hours. Two alterations have been made in the procedure described by Reddy (1954). The first employs the suggestion of Smith, Mellinger, and Patti (1954), who found that the use of 56% instead of 62% sulphuric acid, allowing a longer incubation time, reduced blank values and increased colour development. The second modification involves a simplified method

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for the purification of the commercial butanol. One litre of butanol ("analar") was acidified to *p*H 1 with 50% sulphuric acid. One hundred milligrams of recrystallized phenylhydrazine was then added and the mixture heated to 60° C. for 30 minutes. At the end of this time the temperature was raised to boiling point and the butanol distilled at 117° C., discarding the first 50 ml. Urinary 17-hydroxycorticoids estimated by this method, using cortisone as a standard, showed in 50 individuals a normal range of 3 to 10.2 mg. per 24 hours (mean 6.3 mg.).

Urinary 17-ketosteroids were estimated by the standard method proposed by the Medical Research Council Committee on Clinical Endocrinology (1951).

Results

Normal Response to Corticotrophin. — The administration of corticotrophin zinc to 24 patients with no clinical evidence of adrenal cortical disease resulted in an unequivocal rise in urinary 17-hydroxycorticoids in every case, although there were considerable differences both in the speed and magnitude of the response. The initial control values ranged from 3 mg. to 9.3 mg., and the increase ranged from 6.6 mg. to 27.6 mg. (mean 16.1 mg.) during the day of corticotrophin and from 19.4 mg. to 59 mg. (mean 36.6 mg.) during the following day. One other normal female was not included in this series because she had been receiving small doses of corticotrophin twice weekly for several months, elsewhere, on a mistaken diagnosis of hypopituitarism. Her initial level was 8.1 mg. and after the corticotrophin test there was an enormous increase of 35.4 mg. and 90.9 mg. on the successive days. This result agrees with the finding of Forsham, di Raimondo, Island, Rinfret, and Orr (1955) that repeated small amounts of corticotrophin increase the sensitivity of the adrenal to subsequent stimulation by larger doses.

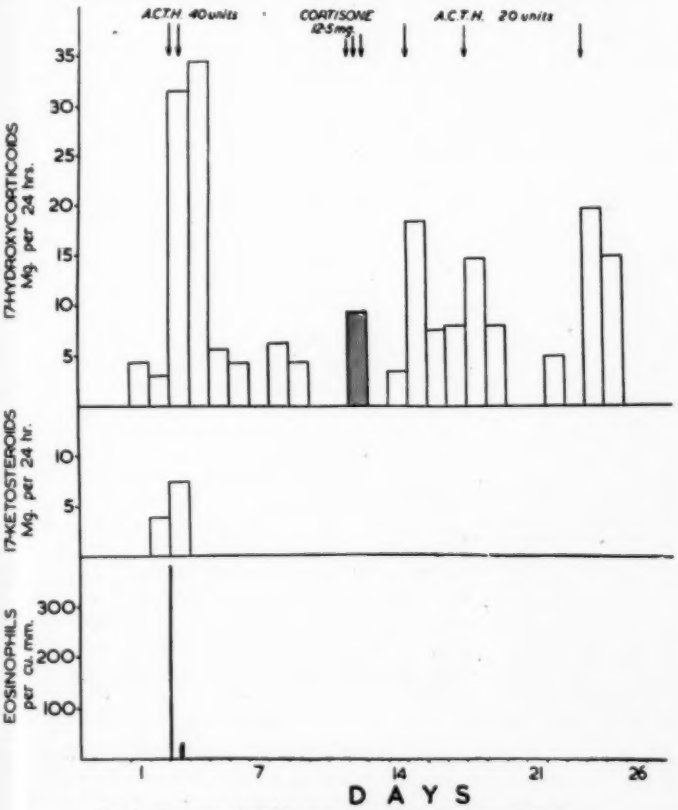

FIG. 1.—Response of normal woman, aged 59, to corticotrophin zinc

In Fig. 1 the responses of the eosinophils, the 17-ketosteroids, and the 17-hydroxycorticoids have been compared in a normal person. It will be seen that in this particular case a definite fall in eosinophils was obtained, but the rise in the 17-ketosteroid level was much less impressive than the rise in 17-hydroxycorticoids. Fig. 1 shows that even smaller doses of corticotrophin zinc cause a rise in 17-hydroxycorticoids lasting from 24 to 48 hours. The effect of oral cortisone, 12.5 mg. three times daily, is included for comparison.

Addison's Disease. — The Table and Fig. 2 show the results obtained when the test is carried out on patients suffering from Addison's disease. Three cases were studied, of which one patient, J. H., was untreated at the time of investigation. This patient showed most of the clinical features of Addison's disease, and the 17-hydroxycorticoid excretion was 2.4 mg. The administration of corticotrophin caused no rise in either the 17-hydroxycorti-

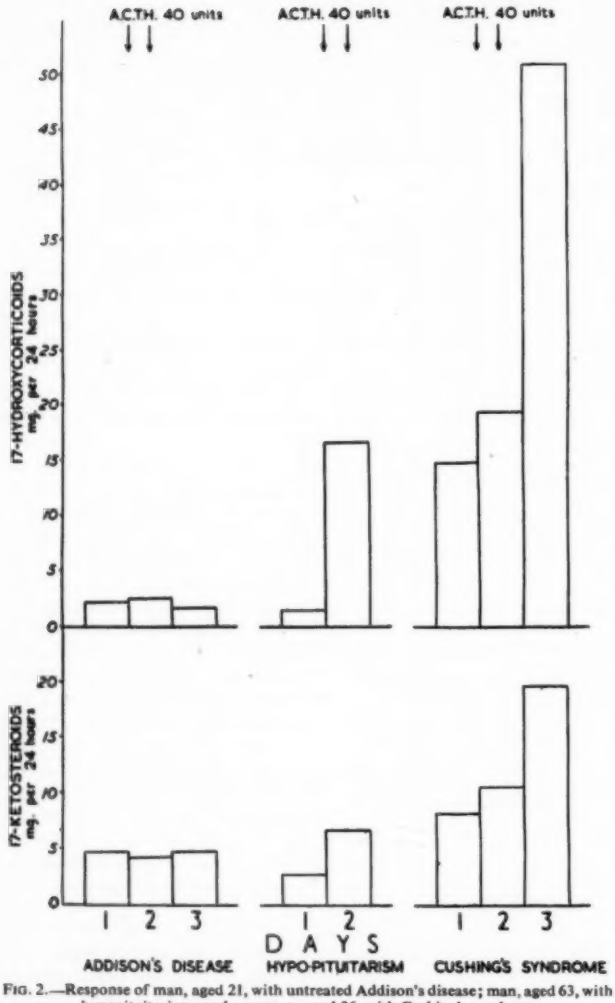

FIG. 2.—Response of man, aged 21, with untreated Addison's disease; man, aged 63, with hypopituitarism; and a woman, aged 26, with Cushing's syndrome.

corticoid or 17-ketosteroid levels. The remaining two patients had suffered from Addison's disease for several years and were receiving treatment with oral cortisone, 12.5 mg. twice daily. This dose was sufficient to produce normal 17-hydroxycorticoid excretion under ordinary conditions, but after corticotrophin there was no significant alteration.

Hypopituitarism.—The use of the 17-hydroxycorticoid test in differentiating between the primary adrenal failure of Addison's disease and adrenal insufficiency secondary to hypopituitarism is shown in Fig. 2 and the Table. A male patient aged 63 suffering from hypopituitarism showed the low 17-hydroxycorticoid excretion of 1.5 mg., but this level rose to 16.5 mg. following corticotrophin. In contrast, the 17-ketosteroids rose only from 2.7 mg. to 6.6 mg. A second male patient also showed a good response, the increase in 17-hydroxycorticoids being 9.9 mg. on the day of corticotrophin and 12 mg. on the following day, although the corresponding rise in 17-ketosteroids was only 1 mg. and 1.4 mg. on these two days. A female patient with long-standing hypopituitarism following post-partum necrosis of the anterior pituitary showed a more sluggish reaction, no rise occurring on the day of corticotrophin, but a rise of 5.6 mg. was obtained on the following day. While an unequivocal increase in 17-hydroxycorticoids was obtained in all three patients using the standard test, the delayed response occurring in the third patient indicates that in cases of suspected hypopituitarism it would be wise to prolong the administration of corticotrophin for three days in order to ensure adequate stimulation.

Cushing's Syndrome.—The application of the test to two patients suffering from Cushing's syndrome due to bilateral adrenal cortical hyperplasia is also shown in Fig. 2 and the Table. In these cases the control 17-hydroxycorticoid values were higher than normal, and after corticotrophin a considerable response was obtained, indicating that the already hyperplastic adrenal cortex was capable of being further stimulated by exogenous corticotrophin.

Discussion

The results shown above indicate that the response of urinary 17-hydroxycorticoids after the administration of corticotrophin zinc differentiates between individuals with normal adrenal cortical

TABLE

EFFECT OF CORTICOTROPHIN ZINC ON URINARY 17-HYDROXYCORTICOIDS IN ABNORMALITIES OF ADRENAL CORTEX

Case	Age	Sex	Diagnosis	17-Hydroxycorticoids		
				1st Day (Control) (mg./24 hr.)	2nd Day (A.C.T.H.) (mg. Change per 24 hr.)	3rd Day (mg. Change per 24 hr.)
J.H.	21	M	Addison's disease	2·4	+0·2	−0·8
V.W.	18	M	Addison's disease (treated)	6·0	+1·9	
I.P.	46	F	,,	3·6	+1·3	
W.P.	63	M	Hypo-pituitarism	1·5	+15·0	
S.W.	59	M	,,	2·4	+9·9	+12·0
J.B.	40	F	,,	2·0	−0·1	+5·6
C.L.	26	F	Cushing's syndrome	14·3	+5·0	+36·4
P.S.	26	F	,,	20·1	+8·7	+18·3

function and those suffering from various abnormalities of the adrenal cortex. The lack of response in Addison's disease is contrasted with the increase obtained in hypopituitarism although this may be delayed in some patients. In the cases of Cushing's syndrome which were investigated, the increase in 17-hydroxycorticoids after the giving of corticotrophin suggested that the patients were suffering from adrenal cortical hyperplasia rather than a malignant tumour, although laparotomy was necessary for confirmation of the diagnosis. Jenkins *et al.* (1955) have obtained similar results, using intravenous infusion of corticotrophin or intramuscular corticotrophin gel. These authors conclude that a rise in urinary 17-hydroxycorticoids of greater than 5 mg. per 24 hours is the best index of normal adrenal cortical response.

The injection of 40 units of corticotrophin zinc 12-hourly provides, in a convenient form, the sustained intensive stimulus required for adequate adrenal function tests and it appears that this preparation has a more prolonged action than corticotrophin gel when it is given in equivalent doses to the same patient (personal finding). Moreover, no anomalous results due to resistance to intramuscular corticotrophin necessitating intravenous infusion have so far been encountered with the zinc preparation.

Nabarro (1954), using corticotrophin gel, has suggested that there is a place for the eosinophil count and the urinary sodium/potassium ratio as screening tests before carrying out the more time-consuming urinary 17-ketosteroid estimation. The limitations of the eosinophil count have already been mentioned and the urinary sodium/potassium ratio may be affected by variations in the diet unless this is rigidly controlled. With the development of a method for the estimation of urinary 17-hydroxycorticoids which is more rapid than the 17-ketosteroid estimation and which provides a more sensitive index of adrenal cortical response, there appears to be less need for the indirect tests.

Summary

An increase in urinary 17-hydroxycorticoids is a sensitive index of adrenal cortical response to corticotrophin.

Patients with Addison's disease show no increase in 17-hydroxycorticoids whereas in hypopituitarism there is a definite response.

In Cushing's syndrome due to adrenal cortical hyperplasia there is a good response to corticotrophin.

Corticotrophin zinc provides a sustained, intensive stimulation of the adrenal cortex in a convenient form.

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STUDIES ON FIBRINOLYSIN USING A CLINICALLY PRACTICAL METHOD OF QUANTITATIVE DETERMINATION

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Methods of quantitative measurement of fibrinolysin are available (Astrup and Alkjaersig, 1952; Loomis, George, and Ryder, 1947; Shulman and Tagnon, 1950). For the most part, these are fairly complicated techniques suitable for the research laboratory but not for the routine clinical laboratory. The method most generally used in the clinical laboratory is a simple qualitative technique in which the blood clot is incubated overnight at 37° C. However, this method may give false positive results in patients with fibrinogenopenia as seen, for example, in post-partum cases (Weiner, Reid, and Roby, 1950). Fibrinogenopenia has at times been misdiagnosed as fibrinolysin because of this error. The qualitative fibrinolysin test will also give false negative results when the enzyme is present in excess but is not strong enough to cause complete lysis of the clot. In these cases, Macfarlane (1937) has recommended using dilute plasma to bring out the fibrinolytic activity. While this method is an improvement over the blood-clot incubation technique, it may still give false positive and false negative results. Furthermore, it gives only a qualitative measure of the defect. Bidwell (1953) and Bidwell and Macfarlane (1951) have attempted to convert Macfarlane's qualitative technique to a quantitative one, and the method now presented is essentially a modification of this method. The quantitative method to be described is useful for any clinical laboratory that is able to do fibrinogen determinations. It has been applied to the routine investigation of coagulation problems, and in addition has been used to study the characteristics of the fibrinolysin in the plasma of a patient with carcinoma of the prostate.

Methods

Defibrinogenation of Plasma.—This was carried out in one of two ways: (1) The plasma was heated for three minutes at 56° C. and was centrifuged for five minutes at 1,000 r.p.m. Then the supernatant defibrinogenated plasma was separated. (2) One hundred units of thrombin were added to each millilitre of plasma to be defibrinogenated. The clot which formed was squeezed out with a glass rod and removed, leaving behind the defibrinogenated material.

Activation of Pro-fibrinolysin.—Two methods were used. The chloroform technique (Tagnon, 1942) for activation of pro-fibrinolysin consisted of adding 0.1 ml. of chloroform plus 0.04 ml. of 0.5 M $CaCl_2$ to each millilitre of plasma. The mixture was incubated at 37° C. for 30 minutes, then centrifuged. The chloroform layer was removed. The residual serum was kept uncovered overnight in the refrigerator, and was ready for use the next morning. The varidase method (Loomis, 1955) consisted of adding 0.1 ml. of varidase (10,000 units of streptokinase and 2,500 units of streptodornase) to each millilitre of plasma. The mixture was incubated for one hour at 37° C.

Purification of Commercial Fibrinogen.—Bovine fibrinogen (Armour) and Cohn's fraction I (obtained from the American Red Cross) prepared from human plasma were used as the starting material. The purification was carried out by Laki's technique (1951).

Fibrin Determination.—Fibrin levels were measured by tyrosine determination using Folin–Ciocalteu (1927) phenol reagent as described by Quick (1951). One millilitre of plasma was diluted to 25 ml. with saline or distilled water plus 100 units of thrombin. The resultant clot was incubated at 37° C. for various periods of time as noted below. In the routine fibrin determination, the clot was removed from the 25-ml. mixture by winding around a glass stirring rod. This method was not adequate for fibrinolysin studies, since the clots, especially after long incubation, were extremely fragile. Consequently, fibrin was separated by filtration through a glass wool filter. The glass wool-clot mixture was squeezed out and washed repeatedly with 0.9% saline, and the fibrin content measured by the routine colorimetric technique of tyrosine determination.

Source of Plasma with Excess Fibrinolysin.—Throughout the work reported in this paper, the actively fibrinolytic plasma was obtained from a 70-year-old man who had carcinoma of the prostate. This patient was first seen because of a bleeding tendency manifested by recurrent spontaneous purpura, finally culminating in a cerebral haemorrhage. He had a bleeding time of 23 minutes (Duke), a clotting time of 8 minutes (Lee, White), a prothrombin content of 60%, and a platelet count (Brecher) of 142,500 per c.mm. A routine fibrinolysin test consisting of 24-hour incubation of whole blood was weakly positive, since the incubated whole blood clot showed only slight breakdown. However, quantitative studies revealed a definite fibrinolytic activity. This patient would probably fit into the group of cases called "fibrinolytic purpura" (Stefanini, 1952).

Procedure.—The technique recommended for measurement of fibrinolysin in clinical laboratories is as follows:

1. Heat fresh normal plasma and test plasma to 56° C. for three minutes. Cool. Centrifuge. Save supernatants (defibrinogenated plasma).
2. Add 1 ml. of normal plasma to each of four tubes labelled *A*, *B*, *C*, and *D*.
3. To tubes *A* and *B* add 1 ml. of defibrinogenated normal plasma. To tubes *C* and *D* add 1 ml. of defibrinogenated test plasma.
4. Bring total volume in each tube to 24 ml. with saline. Place all four tubes in incubator at 37° C.
5. Add 1 ml. of thrombin (100 units per ml.) to each tube.
6. After 15 minutes' incubation, take tubes *A* and *C* from water bath. Filter through glass wool. Wash glass wool-clot mixture repeatedly with distilled water. Squeeze out excess moisture.
7. Do tyrosine determinations on both clots.
8. After 24 hours of incubation, carry out steps 6 and 7 on tubes *B* and *D*.
9. Final results are expressed in terms of percentage loss of fibrin.

Results

Experiment 1.—In this experiment, 20 tubes were each filled with 24 ml. of saline. The tubes were divided into two sets of 10 each. To each of the 10 tubes in set 1, 1 ml. of normal plasma was added. One millilitre of fibrinolytic plasma was added to each of the 10 tubes in set 2. All 20 tubes were placed in an incubator at 37° C. and thrombin was added. At intervals as noted in Fig. 1, two tubes—one from each set—were withdrawn from the incubator and fibrin levels were determined.

The fibrin levels, when plotted against time, gave an S-shaped curve both in the normal subject and in the patient with active fibrinolysin. Often fibrinolytic activity is also fibrinogenolytic.

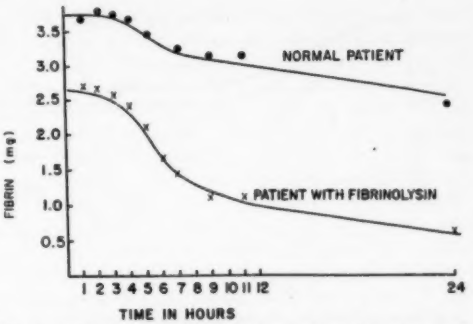

FIG. 1.—Fibrin levels obtained using 1 ml. of test plasma as source of both fibrinogen and fibrinolysin. Amount of fibrin is plotted against time of incubation of clot.

In the case of the patient under discussion, however, the fibrinolysin was sufficiently mild so that his plasma fibrinogen level was only slightly lower than the normal. If his starting fibrinogen level (at time 0) were lower, this comparison between normal and patient could not be carried out as readily (Table I).

TABLE I

EFFECT ON FIBRIN DETERMINATION OF VARYING AMOUNT OF SUBSTRATE

Tube No.	Defibrinogenated Normal Plasma (ml.)	Defibrinogenated Patient Plasma (ml.)	Normal Plasma (ml.)	Total Fibrin Content Stat. (mg.)	Total Fibrin Content after 24 hr. (mg.)
1	0	0	2·5	8·90	6·40
2	1	0	2·5	8·60	5·54
3	0	1	2·5	8·82	4·00
4	0	0	1·0	3·62	3·00
5	1	0	1·0	3·70	2·65
6	0	1	1·0	3·64	1·89
7	0	0	0·5	1·74	1·29
8	1	0	0·5	1·82	1·20
9	0	1	0·5	1·59	0·46
10	0	0	0·25	0·84	0·60
11	1	0	0·25	0·90	0
12	0	1	0·25	0·86	0
13	0	0	0·1	0·20	0
14	1	0	0·1	0·16	0
15	0	1	0·1	0·27	0

Experiment 2.—The results in experiment 1 are dependent on two variables, fibrinogen and fibrinolytic enzyme. In order to control the former, the source of fibrinogen was changed from the test plasma to a normal "substrate." The substrate, in this case, was 1 ml. of normal plasma. The experiment was now set up with 1 ml. of substrate plus 1 ml. of defibrinogenated test material. In this experiment, the defibrinogenation was carried out by heat at 56° C. for three minutes. The results with various test materials are shown in Fig. 2, in which a time

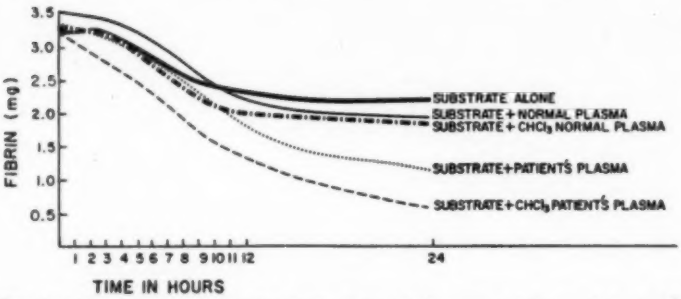

FIG. 2.—Fibrin levels obtained using 1 ml. of normal plasma as substrate and 1 ml. of defibrinogenated test material as source of fibrinolysin. Amount of fibrin is plotted against time of incubation of clot.

curve was obtained. In Fig. 3 a similar series of samples is demonstrated, but this time only two tubes were set up with each; one was tested after 15 minutes' incubation to give the starting fibrin levels, the other after 24 hours.

TABLE II

EFFECT ON FIBRIN DETERMINATION OF VARYING THE AMOUNT OF DEFIBRINOGENATED NORMAL PLASMA

Tube No.	Defibrinogenated Normal Plasma (ml.)	Normal Plasma (ml.)	Fibrin Stat. (mg.)	Fibrin at 24 hr. (mg.)
1	5·0	1	3·58	0
2	2·5	1	3·64	0
3	1·0	1	3·70	2·70
4	0·5	1	3·62	2·89
5	0·25	1	3·70	2·99
6	0·15	1	3·62	3·10
7	0·0	1	3·66	3·18

Experiment 3.—In this experiment the effect of varying the amount of substrate was tested. When the substrate fibrinogen level was low enough even a normal amount of fibrinolysin sufficed to produce complete lysis in 24 hours (Table I).

Experiment 4.—In this experiment the effect of varying the amount of test material was determined (Tables II and III). In Table II it is seen that when the amount of test material was large enough even normal concentrations of fibrinolysin produced complete lysis.

Experiment 5.—In this experiment purified bovine fibrinogen prepared by Laki's technique instead of normal plasma was used as substrate. The results obtained are shown in Table IV. A similar result was obtained using purified human fibrinogen (also prepared by Laki's technique).

In Table V are presented the results of the proposed method of determining fibrinolytic activity applied to the plasma of 23 normal subjects. The drop in fibrin content during 24 hours' incubation ranged from 3 to 40% of the original amount.

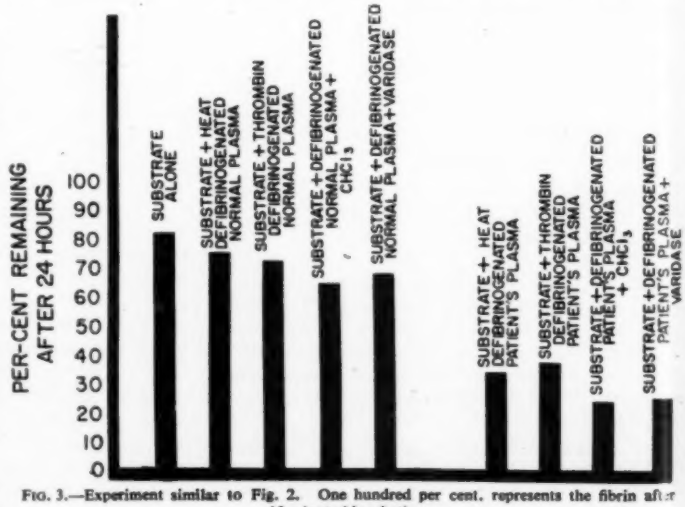

FIG. 3.—Experiment similar to Fig. 2. One hundred per cent. represents the fibrin after 15 minutes' incubation.

Discussion

In experiment 1 an example is presented of fibrinolytic activity in the plasma of a normal subject as compared with the activity in the plasma of a patient with carcinoma of the prostate. The result for the normal subject was similar to that obtained in the 23 normal controls similarly tested (Fig. 1 and Table V). Using a qualitative method, Truelove (1953) was also able to demonstrate fibrinolysin in most normal plasmas on 24-hour incubation. Thus, the mere demonstration of fibrinolysis is without pathological significance. The degree of lysis is the important factor. In Fig. 1 it is shown that the

TABLE III
EFFECT ON FIBRIN DETERMINATION OF VARYING THE AMOUNT OF DEFIBRINOGENATED TEST PLASMA

Tube No.	Defibrinogenated Patient's Plasma (ml.)	Normal Plasma (ml.)	Fibrin Stat. (mg.)	Fibrin at 24 hr. (mg.)
1	5·0	1	3·95	0·0
2	2·5	1	3·72	0·0
3	1·0	1	3·63	1·17
4	0·5	1	3·84	1·28
5	0·25	1	3·72	2·00
6	0·15	1	3·63	2·15
7	0·0	1	3·60	2·40

TABLE IV
USE OF PURIFIED BOVINE FIBRINOGEN AS SUBSTRATE
(1 ml. = 4.00 mg.)

Tube No.	Fibrinogen Solution (ml.)	Test Material (ml.)	Fibrin Stat. (mg.)	Fibrin at 24 hr. (mg.)	% Drop
1	1	0	4·06	3·50	14
2	1	1 ml. normal plasma (heat defibrinogenated)	3·94	2·99	24
3	1	1 ml. normal plasma plus varidase	4·27	3·11	27
4	1	1 ml. patient's plasma (heat defibrinogenated)	4·35	2·14	51
5	1 1	1 ml. patient's plasma plus varidase	3·94	1·08	73

TABLE V
FIBRINOLYSIS IN PLASMAS FROM 23 NORMAL SUBJECTS

Fibrin Content (mg.)		Decrease of Fibrin	
After 15 Minutes' Incubation	After 24 Hours' Incubation	(mg.)	(%)
330	240	90	27
474	410	64	13·5
363	345	18	5
439	263	176	40
456	386	70	15
304	292	12	4
350	215	135	39
304	252	52	17
304	292	12	4
370	270	150	27
316	287	29	9
370	265	105	28
310	263	47	15
486	410	76	15
310	298	12	4
304	269	35	12
287	281	6	3
349	269	80	23
252	240	12	5
316	287	29	9
269	246	23	7·5
304	275	29	9·5
333	292	41	12

fibrin content of the clot of the normal patient fell from 380 to 270 mg. per 100 ml., a loss of 30%, while in the patient with fibrinolysin the loss was from 270 to 60 mg., a drop of 80%. Fig. 1 also shows that the curve of loss of fibrin takes a sigmoid form. The first two to three hours of incubation provided very little breakdown of clot. Jacobsson (1955) found a similar lag period which he called the "time lag" of fibrinolysis.

The comparison between normal and abnormal fibrinolytic activity becomes even more important, and perhaps more difficult to assess, when the original concentration of plasma fibrinogen is low. This is a frequent occurrence in patients with abnormal fibrinolytic activity, in diseases of the liver where fibrinogen production may be impaired, and in fibrinolytic states where there may be an associated fibrinogenolysis. Tables I, II, and III clearly show that variations in the relative proportions of substrate (fibrinogen) and enzyme (fibrinolysis) are important. Table I shows the effect of decreasing the amount of substrate. When fibrinogen levels are sufficiently low, as in tube 11, even normal fibrinolysin produces complete lysis of clots. This clearly illustrates the danger of mistaking fibrinogenopenia for fibrinolytic activity. Furthermore, when the amount of normal fibrinolysis is increased (as in tubes 1 and 2, Table II), complete lysis of the clot occurs. This suggests that the difference between the fibrinolysin of a normal patient and of the patient with fibrinolytic purpura is a quantitative and not a qualitative one.

To obviate the variability of results because of differences in the relative proportions of substrate and enzyme we recommend that the concentration of the substrate be controlled by removing the native fibrinogen from the test plasma and from a control plasma. A definite amount of normal plasma is then added to each to provide fibrin as the substrate. This not only controls the amount of substrate but also establishes the same pre-incubation level for both, thus permitting a more valid comparison of the fibrinolytic activity in the two.

The use of normal plasma as substrate is not ideal. Normal plasma, in addition to fibrinogen, supplies many other ingredients among which are pro-fibrinolysin and anti-fibrinolysin. An attempt was made to use bovine fibrinogen purified by the Laki method. The results, shown in Table IV, are similar to those obtained when the substrate was normal plasma (Table II). The 14% breakdown of the fibrinogen solution alone (Table IV) shows that it was not possible to obtain fibrinogen that was free of fibrinolysin. The techniques which separate fibrinogen from other plasma proteins do not rid fibrinogen of fibrinolysin. For this reason, there seems to be no advantage in using the so-called purified fibrinogen as substrate. Human fibrinogen presented a similar problem. It was not possible to show any species specificity of the fibrinolysin.

The method recommended for clinical use differs from that of Bidwell and Macfarlane (1951) in two important points: (1) These workers did not defibrinogenate their test plasma and substitute normal plasma as substrate, and (2) they removed the clot after 24 hours' incubation by means of a glass rod. This latter method has been found to be extremely difficult to use, especially in cases with mild fibrinolysis. Consequently, filtration through glass wool is recommended.

It should be stressed that the plasma used in step 1 should not be kept at room temperature or at 37° C. for too long a period of time before testing, since fibrinolysin is somewhat labile (Jacobsson, 1955; Truelove, 1953) and will decrease in activity on prolonged standing.

A possible criticism of this method may be raised on the grounds that heat defibrinogenation may destroy some of the fibrinolysin. However, Fig. 3 shows that there is no significant difference when defibrinogenation is carried out by heat or by thrombin. Furthermore, the fact that the addition of thrombin does not cause a greater amount of fibrinolysin activity rules out the possibility that the commercial thrombin used could supply significant amounts of fibrinolysin.

Summary

1. A method of quantitative determination of plasma fibrinolytic activity is presented for routine use in the clinical laboratory. This method depends on normal plasma as substrate and defibrinogenated plasma as test material.

2. When substrate fibrinogen is low enough, normal fibrinolysin will cause complete clot lysis.

3. When the proportion of normal fibrinolysin to substrate is increased, complete lysis of clot will occur.

4. Methods for preparing "pure" fibrinogen do not remove the fibrinolysin, so there is no advantage in using "purified" materials in the test.

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A COMMENT ON THE FRACTIONAL INDIRECT DIAZO REACTION

BY

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Laurence and Abbott (1956) published a description of a micro-method for the estimation of serum bilirubin by the indirect diazo reaction. They indicated that two patterns of colour development are obtainable with foetal and adult sera from cases of severe haemolytic disease due to Rh incompatibility and from obstructive jaundice due to blockage of the common bile duct from carcinoma of the head of the pancreas (personal communication). Adult sera were described as exhibiting a rapid change in colour with maximum colour development at approximately 10 minutes of starting the reaction. Foetal sera exhibited a slower change, maximum colour developing at approximately 30 minutes from starting the reaction. Commercial "pure" bilirubin was found to give a pattern similar to that of foetal sera. These differences in behaviour were interpreted by the authors as indicating the existence of different "adult" and "foetal" substances which gave the indirect diazo reaction in sera.

The implications of such a finding are far-reaching with regard to both theoretical and clinical considerations. This study was undertaken to investigate whether different patterns of colour development were obtainable with the indirect diazo reaction using both normal and pathological, adult and foetal sera.

Materials and Method

Samples of adult blood were obtained from healthy normal individuals, from patients with obstructive jaundice, and from patients with haemolytic anaemia. Samples of foetal blood were obtained from umbilical cords at normal deliveries. Samples of pericardial fluids were obtained at necropsy from cases of kernicterus (not attributable to ABO Rh incompatibility) and from cases of congenital jaundice.

The method and reagents were those of Laurence and Abbott (1956). Serial readings were made in an EEL photoelectric colorimeter using a green filter ("O.G.R.1") at minute intervals for 30 minutes. Haemolysis was looked for with the aid of a pocket spectroscope and by naked-eye inspection. No measurements of the actual amount of haemolysis were made.

Results are summarized in Table I.

TABLE I
SUMMARY OF RESULTS

Material	No. of Specimens	Haemolysis		Time of Maximum Colour Development
		Spectroscopic	Macroscopic	
Adult serum:				
Normal	4	Absent	Absent	10 minutes
Pathological ..	9	,,	,,	10 ,,
Umbilical cord serum	10	,,	,,	10 ,,
	4	Present	,,	10 ,,
	5	,,	Present	30 ,,
Pericardial fluid ..	1	Absent	Absent	10 ,,
	1	Present	,,	10 ,,
	2	,,	Present	30 ,,

Results

Unhaemolysed sera obtained from adult normal and pathological cases gave similar patterns in which maximum colour developed at approximately 10 minutes from starting the reaction.

Sera obtained from umbilical cord bloods exhibited two patterns depending on the degree of haemolysis. When haemolysis was present on naked-eye inspection, maximum colour developed at approximately 30 minutes. When haemolysis was absent on naked-eye inspection, and when it was only detectable spectroscopically, maximum colour developed at approximately 10 minutes.

Pericardial fluids behaved like umbilical cord sera, in that the 10-minute or the 30-minute pattern was obtained depending on the degree of haemolysis.

Serial dilutions of haemoglobin were made by adding a solution of haemoglobin obtained by freezing and thawing adult erythrocytes to unhaemolysed adult serum. Concentrations of haemo-

globin not exceeding approximately 3.5 g. per 100 ml. serum produced no change in the pattern of colour development. Higher concentrations changed the pattern from the 10-minute to the 30-minute type. Serial dilutions of the same haemoglobin solution in water without serum gave results similar to those for the dilutions of the haemoglobin solution in serum.

Discussion

The results here presented demonstrate that, with the indirect diazo reaction, maximum colour develops at approximately 10 minutes in the case of both adult and umbilical cord sera, when haemolysis is absent or slight. Naked-eye haemolysis changes the pattern of colour development so that maximum colour occurs at approximately 30 minutes. The pattern due to the bile pigments in adult and foetal sera is similar, and there is no evidence that the pigments of adult and foetal bloods are not identical.

Najjar and Childs (1953) obtained crystalline indirect serum bilirubin from icteric sera from cases of haemolytic anaemia, sickle cell anaemia, haematoma fluid, jaundice of the newborn, kernicterus, and congenital familial non-haemolytic jaundice with kernicterus. All these sera yielded, by the same technique, crystals which had identical physical and chemical properties.

Using the method of "reverse phase" chromatography, Cole and Lathe (1953) separated and extracted the direct-reacting material and the indirect-reacting pigment of serum. The direct-reacting material was obtained from sera of patients with obstructive jaundice and with hepatitis. The indirect-reacting pigment, probably bilirubin, was obtained from sera of cases of haemolytic disease of the newborn attributed to Rh incompatibility and from adult haemolytic anaemia. With both pigments maximum colour developed at approximately 10 minutes of starting the reaction. No differences were described between the adult and foetal pigments.

More recently Cole, Lathe, and Billing (1954) produced additional evidence that the indirect-reacting pigment is bilirubin and separated the direct-reacting material into two pigments with spectral curves similar to, but not identical with, that of bilirubin, and suggest that these pigments probably have a tetrapyrrole structure like that of bilirubin. Lathe (1954) has stated categorically that the predominant pigment of neonatal jaundice is bilirubin and that in nearly all cases examined the sole pigment present during the period of rising concentration was bilirubin, the direct-reacting pigments appearing during the period of falling concentration.

Bilirubin is a product of the metabolism of the haem nucleus of haemoglobin. The haemoglobins of the species studied have been shown to contain an identical haem nucleus, and differences between haemoglobins, normal and abnormal, human and animal, seem to be attributable to differences in the globin moieties. Pauling, Itano, Wells, Schroeder, Kay, Singer, and Corey (1950) showed that the haems of haemoglobins A and S are identical. No evidence is available that the bilirubins arising from haemoglobins A and F are not similar.

It is interesting to note that the level at which added haemoglobin showed a definite effect on the quantitative micro-estimation of Laurence and Abbott (1956) coincides with that at which haemoglobin added to serum or water changed the pattern of colour development in this study.

No attempt was made to study the details of the reaction between haemoglobin and the diazo reagent, though the almost immediate change in colour to a dark brown is probably explained by the conversion of haemoglobin to acid haematin.

Since haemoglobin not only affects the quantitative estimation of serum bilirubin but also interferes with the normal pattern of colour development, inferences made from serial estimation of serum bilirubin in the presence of haemolysis may be misleading.

Conclusions

(1) Unhaemolysed adult and umbilical cord sera exhibit similar patterns of colour development with the indirect diazo reaction.

(2) Adult and umbilical cord sera showing haemolysis on naked-eye inspection exhibit similar abnormal patterns of colour development with the diazo reaction.

(3) No evidence was obtained to indicate the existence of different indirect-reacting pigments in adult and foetal unhaemolysed sera, in normal or pathological specimens.

(4) No evidence is available in the literature that the bilirubin of adult sera is not identical with that of foetal sera.

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TECHNICAL METHODS

A Simple Apparatus for Shaking Bacterial Cultures

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The importance of shaken cultures as compared with stationary cultures is now well established. This paper describes a simple shaker which, it is believed, will be particularly valuable in routine laboratories. It is made of slotted angle "dexion" to fit a suitable water-bath. Owing to its rigid construction it may be removed from the water-bath freeing the latter for its original purpose. The shaker is provided with rubber feet and may be employed standing by itself as an agitator at room or refrigerator temperatures. The cultures may be grown in optical tubes, flasks, or bottles. A brief description of some typical results obtained with this machine is included.

Materials and Methods

The Shaker.—This is illustrated in Fig. 1. In essence it consists of a rigid framework of "dexion" which fits closely over the bath employed, but it is detachable and has rubber feet so that it will stand quite safely on the bench. At one end is a single-speed electric motor which drives an overhead rod. The number of swings per minute is controlled by means of the two pulleys and driving belt. The vessels to be shaken are either clipped on to a bar some 5–6 cm. below the rod or else on to a horizontal plate suspended in the water. This plate carries two rows of Terry clips which are rubber-covered to prevent them scratching the optical glass tubes.

In the machine illustrated the speed in complete cycles/min. is variable from 30 to 90. Above 90 cycles/min. the contents of the bath splash a great deal when large flasks are used. It was decided to use a motor (with a reduction gearing giving a fixed speed), with a condenser in the circuit rather than a variable speed motor (Klaxon, 49, Upper Brook Street, W.1, output torque 4 lb. in., 110 r.p.m.). This was done because it is not uncommon for the electric power to fail and then suddenly return with unfortunate effects on the second type of motor. With the fixed-speed motor one may safely leave the shaker running for days on end without attention.

In order to shake vessels of varying shape it is convenient to be able to vary the stroke. In Fig. 1 "view on arrow B" shows the connexion of the overhead rod with the gear wheel. This connexion is attached to the wheel by means of a milled-headed screw which, it will be seen, can be screwed into any one of eight positions. These alternative positions are dispersed on a curved line from the centre to the periphery.

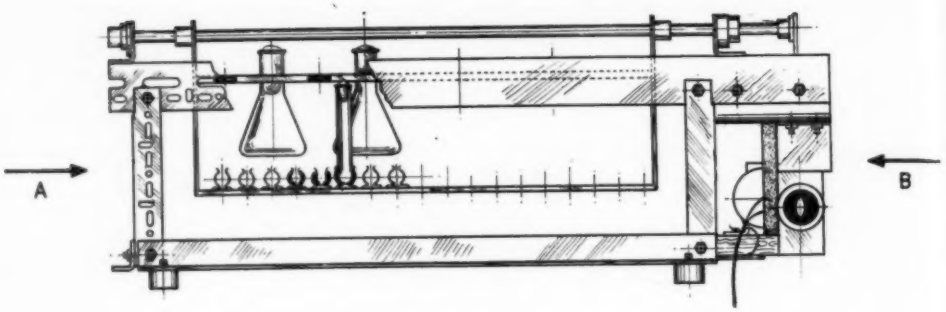
Bacterial Cultures.—In the experiments described here two coliform organisms were employed, namely, motile coli K12 and non-motile coli NCTC 6064. These cultures were usually shaken in the optical glass tubes described elsewhere (Gorrill and Gray, 1956), but equally good results were obtained with conical flasks.

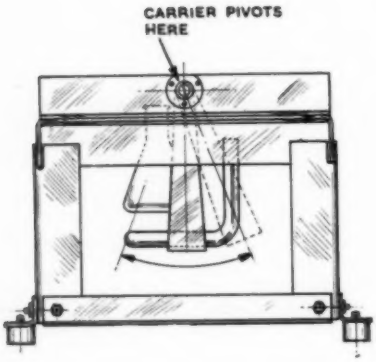
Media.—Good results were obtained with tryptic digest broth from the Southern Group Laboratory, Park Hospital, London, S.E.13. Buffer 56 (Monod, Cohen-Bazire, and Cohn, 1951) was used for making the dilutions.

Counting.—Serial dilutions were made in buffer using 0.1 ml. pipettes and 9.9 ml. volumes of buffer. The pipettes were made from "pyrex" glass tubing internal bore 1 mm. and overall diameter 5–8 mm. The tubing was drawn into blunt-nosed pipettes 20 cm. long. They were stored in copper cans 25 cm. long by 7 cm. diameter. The pipettes were calibrated to contain 0.1 ml. by the use of a weighed quantity of mercury. Tenfold steps were interposed where necessary by using 1 ml. pipettes and 9 ml. of buffer. Counts were made by adding 0.1 ml. of the dilution to 3 ml. of digest broth containing 0.7% agar held at 46° C. When mixed the agar was poured over an infusion agar plate and allowed to set. Only plates with between 40 and 200 colonies were counted. Duplicate dilutions were made and two plates poured from each dilution.

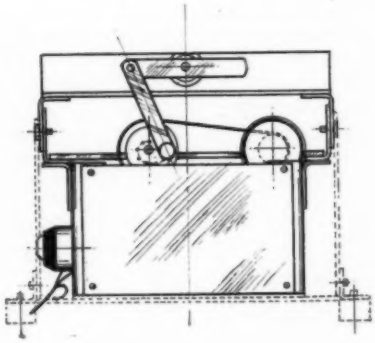
Results

This shaker has been in use for over a year and has shown itself to have considerable practical value. It is capable of producing higher densities of bacteria more quickly than the equivalent stationary culture. This is well shown in Fig. 2, which gives the results of shaking *E. coli* K12 at different speeds compared with a stationary culture. It will be seen that while the rate of shaking between 30 and 90 cycles/min. has little effect on the growth rate, the difference between shaken and unshaken cultures is considerable. Similar types of curves have been produced with a wide range of bacteria. In the experiment illustrated in Fig. 2 the inoculum was derived from an overnight broth culture, but equally good results can be obtained

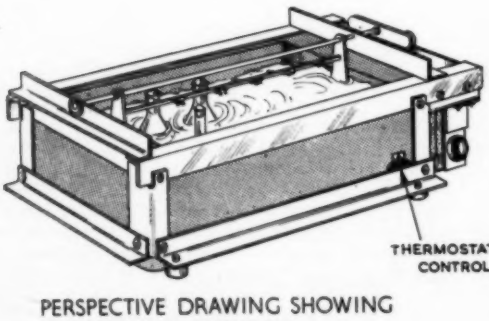




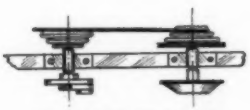
VIEW ON ARROW 'A'



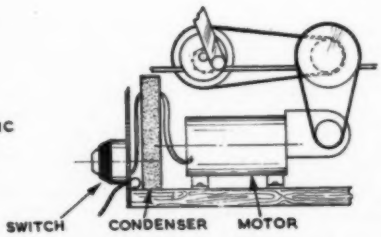
VIEW ON ARROW 'B'



PERSPECTIVE DRAWING SHOWING BATH IN POSITION



PLAN VIEW OF GEARS



END ELEVATION WITH PLATE REMOVED

FIG. 1.—The design of the shaker.

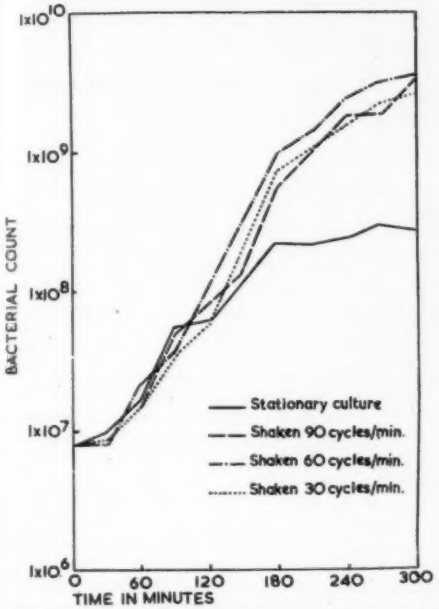


FIG. 2.—The effect of speed of shaking on the growth curves of *E. coli* K12.

when a bacterial colony picked off a plate is used as the inoculum. This is of value in producing broth cultures for subsequent antibiotic sensitivity testing. If the inoculum size is important as it is with some antibiotics, the cultures should be shaken to a standard turbidity before use.

It has been the custom to carry out dilutions and counts using cold (4° C.) buffer, as this prevents further multiplication. The buffer is without effect on the bacteria tested, an important point in view of Wilson's findings (1922, 1935) that many diluents were bactericidal. However, it was possible that during logarithmic growth the actively dividing cells would be susceptible to cold shock (Hegarty and Weeks, 1940), and killed when diluted into ice cold buffer. With the organisms tested the phenomenon of cold shock has not been seen, but it would be as well to test the susceptibility to dilution at 4° C. of any organism before using this method.

Apart from the obvious uses of the shaker there are others that have been developed. Only one will be described here, and that is the maintenance of cultures in the continuous log. phase with a known mean count. The method is illustrated in Fig. 3 using *E. coli* 6064 as the test organism. A series of tubes were set up in the bath containing 9.9 ml. of broth, and the first inoculated with 0.1 ml. of broth culture. Every two and a half hours 0.1 ml. was removed from the growing culture and transferred to the next broth tube. By this means log. cultures may be maintained throughout the working day. The optimum time of transfer and the volume transferred varied with the culture used. Only by using the correct proportions was it possible to maintain the mean count at a steady level.

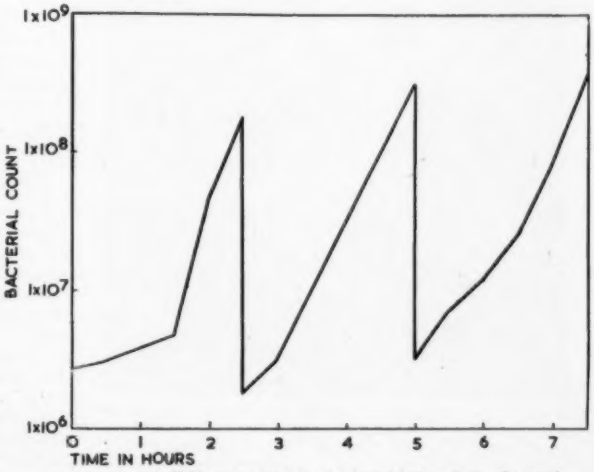


FIG. 3.—*E. coli* 6064 maintained in the logarithmic phase of growth.

Discussion

It is believed that the great merit of this shaker is its adaptability. While being built to the size of an available bath it may be easily modified to fit a new bath owing to the versatility of the "dexion" from which it is made. Similarly, it is strong enough to be lifted off the bath and stacked out of the way when not in use. Finally, it has a number of uses as a shaker at room and refrigerator temperatures.

Antibiotic sensitivity testing is an important part of every routine laboratory's work, and it is undoubtedly important to employ actively dividing cells at a reasonably constant viable count as the inoculum for either plate or tube tests. By the use of this shaker colonies may be picked into broth and the resulting culture tested in the afternoon.

The application of this machine to the production of a continuous log. phase culture has been included to show that it has considerable applications in research.

Summary

A simple, robust shaker designed to fit over an existing water-bath or to function standing alone is described. It is easy to make and may be adapted to individual needs. Some examples of its use have been briefly described.

It is a pleasure to acknowledge the assistance of the workshop at Guy's Hospital Medical School where the shaker was built. Our thanks are due to the Medical Illustration Department who produced the figures.

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Perspex Jars for Pathological Museums

L. W. PROGER

From the Royal College of Surgeons, London

(RECEIVED FOR PUBLICATION NOVEMBER 23, 1956)

During the past 10 years "perspex" has become the accepted material for constructing jars for the display of pathological specimens. While it is now possible to embed specimens in solid blocks of transparent plastic, the jar containing fluid continues to be the popular choice. The elasticity of the material allows the jar to be completely filled with fluid giving an effect of solidity, and the specimen can easily be removed for further histological examination at any time.

A plastic jar to be satisfactory should be easy to make, remain free from leakage, and be perfectly rectangular so as to take full advantage of the optical properties of the material.

Many of the early difficulties in construction have been due to failure of cemented joints, since the nature of the material makes it almost impossible to obtain machined surfaces of sufficient accuracy to employ simple butt joints. The method to be described has avoided these difficulties and the jars have stood rough usage for up to 10 years without evidence of breakage.

For permanent joints in large containers which are submitted to considerable stress, the makers recommend their cold welding process. In this process the joint is filled with a methacrylate dough which is then polymerized by exposure to ultra-violet light. The resulting join has similar properties to the parent material (Imperial Chemical Industries Ltd., Plastic Division, I.S. Notes, 343 and 141).

Method of Construction and Materials

The method depends on the use of rebated joints for the body of the jar. This type of joint enables the solvent to correct minor irregularities in the cut edges and checks any tendency for the plastic sheet to bend while the cement is hardening. In order to obtain sufficient accuracy, the pieces for the jar are first cut out roughly with a band saw and then trimmed accurately to size by removing a thin shaving with a trimming cutter. In this way heat distortion is reduced to a minimum.

Since perspex is a bad conductor of heat, the cutting tools should be sufficiently heavy to conduct the heat away from the cutting edges. The use of fine-toothed slitting saws should be avoided.

Perspex is supplied in convenient stock sheets measuring 3×4 ft., and three thicknesses cover most requirements for the usual museum jar size: 3/16 in. for the body of the jar, ¼ in. for the top and bottom, and 1/16 in. for the specimen supporting panels, which may be clear, opaque, or coloured as required.

The milling equipment is mounted on a bench with a working top made of ¼ in. duralumin plate 4×2 ft.

It has been found more convenient to use separate cutters accurately adjusted for each operation than a standard type milling machine which requires resetting for each operation (Fig. 1).

Standard high-speed steel milling cutters give satisfactory results and can be easily reground or replaced. For large-scale production special cutters for plastics would be more suitable.

Cutters.—The descriptions of the cutters required for the four separate milling operations are as follows:

Panel Trimming.—Side and face cutter, diameter 4 in., width ¼ in., hole 1 in., mounted with 5/16 in. of the cutter protruding through a slot in the centre of the bench top. The position of the face of the cutter is marked by a line inscribed on the surface of the bench, and this is used in conjunction with an adjustable straight-edge guide.

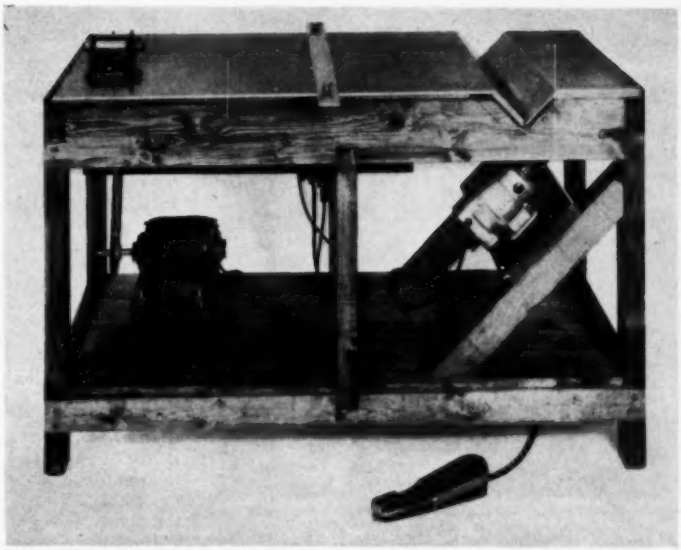

FIG. 1.—Photograph of the milling bench. The rebate cutter is on the left. The adjustable straight-edge and trimming cutter towards the centre, and the corner rounding cutter on the right.

Rebate Cutting.—Side and face cutter, diameter 3 in., width ¼ in., hole 1 in., mounted on a fixed straight-edge guide above the bench with 3/32 in. clearance between the cutter and the bench top, and the cutter protruding 5/32 in. beyond the guide.

Specimen Panel Groove.—Side and face cutter, diameter 3 in., width 3/32 in., hole 1 in. Mounted in place of the rebate cutter with back spacing washers to locate the groove in the desired position.

Corner Rounding.—Corner rounding cutter (single), diameter 2½ in., radius of circle 3/16 in., hole 1 in., mounted in a right-angled trough guide made of ¼ in. duralumin plate, and adjusted to cut a full quarter circle from the corners of the jar.

The cutters are mounted on high-speed mandrels carried in ball races and driven by V belts and pulleys. The trimming and rebate cutters are driven through a countershaft by a 0.4 h.p. 2,850 r.p.m. motor controlled by a foot switch. The corner rounding cutter has an independent motor, since the countershaft was not conveniently placed in this instance. All cutters are provided with safety guards detachable for cleaning.

Cementing and Fixing Joints.—Chloroform is the most convenient laboratory solvent for cementing. The edges to be fixed are first soaked in the solvent for two to three minutes and then held together with a light, even pressure. It is important to localize the action of the solvent to the joint, and this is done by resting the edge of the panel on a piece of plate glass and applying the solvent with a Pasteur pipette. The adjacent surfaces

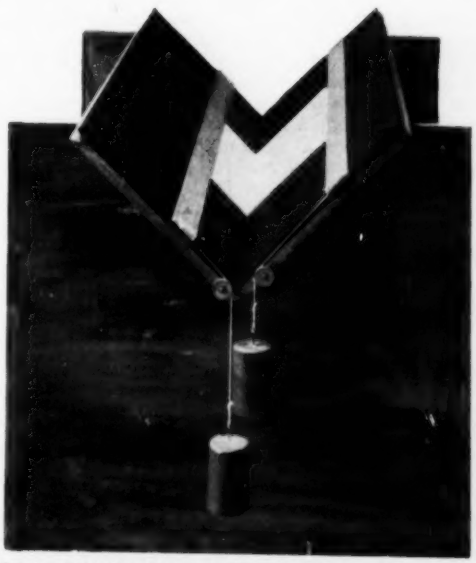

FIG. 2.—The holding frame, showing two panels pressed together by weighted bars. The edges of the larger panel are rebated. (The glass plates in the trough have been masked with black paper to avoid reflections.)

FIG. 3.—The trimming cutter, from which the safety guard has been removed to show the inscribed line and the adjustable straight-edge.

of the panel are protected from the action of chloroform vapour by the application of strips of "sellotape." After softening the edges of two panels, they are lightly pressed together and placed in a frame which holds them in the correct position until the cement has hardened. The holding frame (Fig. 2) consists of a right-angle trough 2 ft. long and 1 ft. deep, made of ½ in. ply board and lined by ¼ in. glass plates to give smooth flat surfaces. Weighted bars are placed on the free edges of the panels so as to bring the cemented edges together with a light, even pressure, at the same time holding them at right angles. The joint is sufficiently firm for removal after 10 to 15 minutes.

The need for accuracy in cutting the panels cannot be overemphasized. Irregularities cannot be made good either by prolonged soaking in solvent or by the local application of cramps.

Sequence of Operations.—First cut out roughly from the stock sheet the four panels for the front, back, and two sides of the jar.

Set the adjustable straight edge approximately 1/16 in. wider than the required size and trim one edge of a pair of panels (Fig. 3). Reset the straight edge to the exact size and trim the opposite edges of the panels. Trim the other pair of panels. The top and bottom edges of the panels are not trimmed at this stage. Cut the rebates on the edges of the front and back panels. This cutter should remove a strip 5/32 in. wide and 3/32 in. deep (Fig. 4).

Cut the locating grooves for the specimen supporting panel on the inner surfaces of the side panels of the jar.

Wash the panels in warm soapy water to remove grease and dust. Apply "sellotape" along the edges to be cemented.

Soak the edge of a side panel and the rebated edge of a front or back panel in chloroform for two to three minutes, place in the holding frame and adjust the weighted bars to give a firm, even pressure. After 15 minutes repeat with the other pair of panels. The four free edges of the two pairs of panels are now soaked in solvent and reassembled in the holding frame. In this case pressure is applied to the uppermost joint by a weighted bar of right angle section. The body of the jar is now complete, and the joints are allowed to consolidate by the evaporation of excess solvent. Consolidation may be hastened by placing the jar in an oven at 30° C. overnight. Protective tapes are removed from the inner surfaces of the jar.

The irregular top and bottom edges of the jar are now cut off square on the band saw. Any saw marks are removed by lightly rubbing on fine glass paper attached to a flat glass plate.

The corners are rounded with the corner rounding cutter (Fig. 5). Top and bottom panels are cut slightly oversize from ¼ in. sheet. The top has a ⅛ in. filling hole drilled in one corner, the bottom is preferably made of opaque white "perspex" to improve light reflection and to prevent scratch marks from showing through.

The bottom edges of the jar are soaked in chloroform, placed on the bottom panel and held in place with a suitable weight until fixed.

FIG. 4.—The rebate cutter; note the length of the mandrel and retaining screw, which allows the panel groove cutter to be placed at the required distance from the guide.

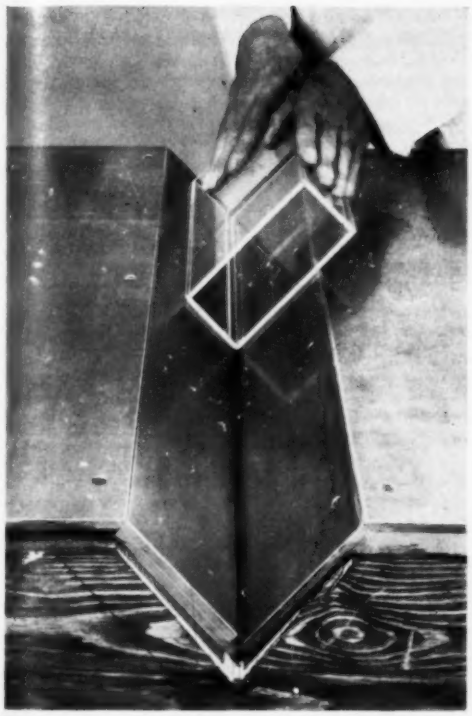

FIG. 5.—The corner rounding cutter, on which the right-angle trough ensures an accurate cut.

The top edge of the jar is soaked in solvent and the panel carrying the specimen slipped into locating grooves cut in the sides of the jar. The jar is then filled with mounting fluid to within ½ in. of the top and the top panel placed in position. When the joint has hardened, the jar is topped up with fluid and the filling hole sealed with a perspex plug dipped in solvent. Trapped air bubbles are often troublesome and may be freed with the aid of a suction pump.

The top and bottom edges are passed over the corner rounding cutter and the filling plug smoothed off with a fine file.

The machined corners are rubbed down with fine glass-paper and the resultant matt surface polished with metal polish and finished with " perspex " polish No. 3.

Mounting the Specimen.—Specimens are conveniently mounted on a separate " perspex " panel before being placed in the jar. They may be attached by looped threads passed through double holes (pierced in the panel with a hot wire) and tied at the back of the preparation. It may be convenient to cut shaped holes in the panel to accommodate solid projections in some specimens, or to suspend the specimen in a rectangular frame, and 1/16 in. is a suitable thickness for most supporting panels, which may be clear or coloured as required.

The panel or frame carrying the specimen is positioned in the jar by sliding it into shallow vertical grooves which have previously been cut in the inner surfaces of the side panels. When the jar is filled with fluid the supports become almost invisible.

Illustrations.—It is sometimes instructive to include a photomicrograph, clinical photograph, radiograph, or explanatory diagram with the specimen, and these may be mounted on an opaque white panel slipped into grooves behind the specimen panel. If photographic prints are soaked in chloroform for a few minutes, sufficient solvent is taken up by the paper backing to enable the print to be stuck to the plastic surface. The photographic emulsion is not affected by the usual glycerine mounting solutions.

Labelling.—Museum jars are usually provided with reference numbers. Paper labels printed on a small hand press are inexpensve and reasonably satisfactory. They may be attached to the jar after dipping in chloroform and given a protective coat of varnish. Engraved labels are more permanent. With the aid of a small bench type engraving machine, labels may be cut out of 1/16 in. white perspex and pressed into a corresponding recess cut in the front of the jar. These labels, being flush with the surface of the jar, are easily cleaned, and can be prised out of the recess and replaced in the event of a change of number being necessary.

An Automatic Staining Machine Designed for Staining Blood Films Made on Coverslips

WILLIAM M. DAVIDSON, F. R. BAREHAM, L. D. KITCHEN, AND S. H. PEGG

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(RECEIVED FOR PUBLICATION FEBRUARY 6, 1957)

In most forms of microscopical investigation some form of routine staining is essential. Many such procedures are merely repetitive yet are time consuming. A machine to lift the preparations from staining pot to staining pot at predetermined intervals is not a luxury but a basic requirement in a laboratory where a simple staining method is used 50 or more times a day.

Originally conceived to relieve the laboratory staff of unnecessary routine work, the present machine was expected to produce staining results of reasonable quality. The standard achieved has been far beyond expectation and for the past six years many thousands of films have been stained to a uniform standard, otherwise attained only by the personal care of an experienced technician.

In the machine a central shaft carries 12 radial arms. The ends of these arms come to rest over the centre of each reagent pot. The blood smears are made on $\frac{7}{8}$ in. square No. 2 coverslips, by sliding apart two of these after a drop of blood between them has been allowed to spread almost to its full extent by capillary attraction (Ehrlich and Lazarus, 1900 ; Wintrobe, 1956). After drying, the coverslips are placed in perspex containers which are attached to swivel hooks at the ends of the arms. Loose perspex discs above the hooks cover the pots during the time of immersion to reduce evaporation. At predetermined intervals the central shaft is raised by an electric motor until the slide carriers are clear of the pots. At this point a second motor is brought into action and rotates the arms in a counter-clockwise direction until each is above the next "station." The first motor then takes over again and lowers the carriers into the solutions, the whole movement being completed in under 20 seconds. The staining period is determined by an electronic timer which can be set at intervals between a half and three minutes. In this way the preparations are passed through the successive solutions and are finally removed by striking a ramp at "station twelve." Apart from loading the carrier and attaching it to the hook at "station one" the process is entirely automatic, and as arranged the machine has a maximum staining capacity of 120 coverslips hourly.

A range of immersion times, from a half to over 12 minutes, can be predetermined by varying the setting of the timer and the number of pots used for any reagent. In this laboratory the Jenner–Giemsa method is in routine use for the staining of human and animal blood films. The reagents are contained in standard ointment pots with an internal diameter of 6 cm., a depth of 4 cm., and a capacity of 80 ml. The timer is set at two minutes. Absolute methyl alcohol in two pots gives four minutes for fixing. Three pots of Jenner's stain, diluted in an equal quantity of *p*H 6.8 phosphate buffer, give optimum staining of the red cells and the cytoplasm of the leucocytes; and a further six minutes in Giemsa stain, diluted 1 in 10 in *p*H 6.8 phosphate buffer, enhances the staining of the chromatin of the nuclei and the granules in the neutrophils and platelets. Finally, two pots of buffer solution, aerated to keep up a constant washing action, remove any scum which has formed. After passing through the buffer, the films in their carrier strike the ramp and come off the machine. They are normally allowed to dry in air, but a quick rinse in distilled water, followed by blotting dry, may improve the final differentiation of the structural details.

In staining coverslip spreads made from tiny fragments of bone marrow, the longer time necessary (12 minutes) is achieved by sending the preparations twice through the Giemsa stain pots.

With the reagents detailed it costs 1s. 6d. to load this machine against approximately 1s. for a hand method using four Coplin jars.

Although this machine, as originally developed and constructed by the Physics Laboratory of King's College Hospital, has worked satisfactorily for over six years, it has long been evident that it could be simplified. It contains two electric motors, several relays and many other electrical components, in addition to the mechanical parts for the lifting and turning. Further, the two motors require perfect synchronization and have to be provided with a braking mechanism for efficient operation. In a newly designed modification mechanical and electrical simplification has been achieved. Only one motor is required to operate the lifting and turning mechanisms which are mechanically linked and cannot fall out of synchronism. After designing the basis of the simplified machine and producing a working model of the mechanism, it seemed unwise to incur further development costs and the idea has been passed to a commercial firm (Elliotts Liverpool, Ltd.).

This mechanical aid to staining has proved of the greatest value in the laboratory routine. The uniformly high quality of the staining attained has made it possible to place greater weight upon fine changes in the details of the cell morphology.

Stains Used

1. Jenner and Giemsa.
2. Buffer *p*H 6.8 in form of phosphate buffer tablets.
3. Methyl alcohol (maximum acetone 0.05%).
4. Glycerine B.P.

Jenner is used as a 0.5% alcoholic solution, and Giemsa as a 0.76% solution in equal parts of glycerine and alcohol.

Summary

An automatic machine for the staining of microscopical preparations is described. This machine, built in the hospital, has been in successful routine use for six years. Future developments are indicated.

Designed originally to save time and labour, it has proved of even greater worth in the uniformly high quality of the staining achieved.

Such a machine is a necessity in a busy laboratory where one staining method is used many times a day.

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An Apparatus for Holding Chromatography Paper During "Spotting-On"

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(RECEIVED FOR PUBLICATION MARCH 19, 1957)

It is often difficult to find a convenient support for the paper when applying the spots of material before chromatography.

The apparatus described here has been in constant use for the past two years. It keeps chromatography paper clean and still during "spotting-on," and also holds it so that a drying current of air or nitrogen can, if required, be continuously applied from above or below.

It consists of two 27 cm. square plates of $\frac{1}{4}$ in. perspex. One sector, 21 cm. × 3 cm., is cut out of each plate in the same position, 3 cm. from one edge. Four L-section legs, 5 cm. long, made from $\frac{1}{4}$ in. perspex, are glued to one plate (Fig. 1). The cost of the perspex is about 12s. 6d., and the apparatus takes a skilled technician about three hours to make.

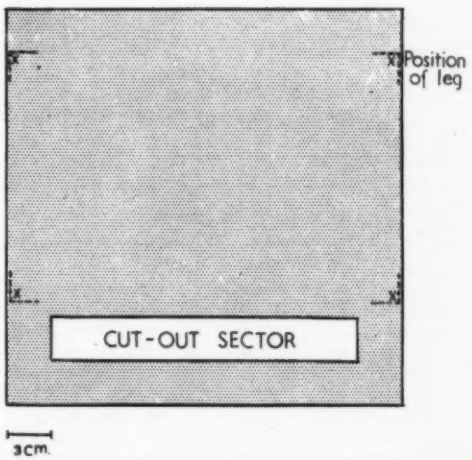

FIG. 1.—Plan of perspex plate (for dimensions see text).

In use the lower plate stands horizontally on its legs. A standard 10 in. square paper is placed symmetrically on it, and the second plate is placed over the paper so that one cut-out sector is above the other, the paper being sandwiched between the

plates (Fig. 2). The material for chromatography is then applied in the normal way to the strip of exposed paper between the sectors.

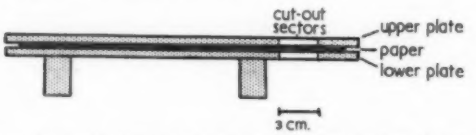

FIG. 2.—Profile diagram of apparatus, showing position of paper sandwiched between the plates.

I would like to thank Mr. W. Steele, F.I.M.L.T., for making the apparatus.

Seventh International Cancer Congress, London, 1958

Those planning to attend the 7th International Cancer Congress, which will take place at the Royal Festival Hall, London, from July 6 to 12, 1958, are reminded that enrolment forms must be received at the Congress Office (45 Lincoln's Inn Felds, London, W.C.2) *by January 1, 1958*, if a late fee is not to be incurred. Registration forms may be obtained from the Secretary-General at that address.

BOOK REVIEWS

Human Blood Coagulation and its Disorders, 2nd ed. By Rosemary Biggs and R. G. Macfarlane. (Pp. xxi +476; 53 figures. 42s.) Oxford: Blackwell Scientific Publications. 1957.

The literature on blood coagulation is as voluminous as it is incomprehensible to the outsider. The authors say in their opening pages: "It is the modest purpose of this book to attempt to reduce this profusion to the smallest possible residue of useful and sober facts; to sort out the different names which really belong to the same thing, the different things which have had the same name, the things which probably do exist from the things which probably do not, and to present what is known about the probable ways in which the things which remain react together to produce a clot." From this we may deduce that they are masters of the English language and that they approach their subject in the true spirit of Oxford scholarship. Add to this that they lead one of the most active and fertile schools of study in this field; then one has the ingredients of this model of all that a medical textbook should be.

The first half of the book sets out in less than 200 pages our present knowledge of blood coagulation. Beginning with the thrombin-fibrinogen reaction the authors work backwards through prothrombin to the accelerators and thromboplastins that initiate the clotting reaction. Their approach is broadly historical, setting down an ever-growing pile of observations and hypotheses in the order of their discovery, and yet by superb marshalling and masterly selection leaving the reader with a coherent grasp of the substance as well as the complexities of their subject.

The next part of the book describes the clinical states that arise from, or produce, defective clotting. The authors' deep theoretical knowledge of these conditions is clearly supported by great practical experience of their management, although it is the principles rather than the details of treatment that they set out. This section includes a very clear and balanced account of the principles of anticoagulant therapy.

The following chapter, describing the systematic investigation of coagulation defects, has been much clarified and improved. A very useful glossary has been added to this edition (but it omits proconvertin). Finally there is a detailed and authoritative description of technical methods, an excellent index, and a list of some 1,400 references. MARTIN HYNES.

A Practical Manual of Medical and Biological Staining Techniques, 2nd ed. By Edward Gurr. (Pp. xxv +451. 42s.) London: Leonard Hill. 1956.

The good housewife can scarcely see a new cookery book without wishing to buy it, and a wise husband may even forestall her by a gracious gift if the title

seems promising. The good technician and the wise pathologist are likewise interested in books on histological technique. We could justifiably suspect the wife who buys because the cover has a picture of Phillip This or Sidney That, and certainly we do not expect any technician will buy Mr. Gurr's book because the frontispiece shows the author reading in his laboratory.

Most of those who might buy this book will know that Mr. E. Gurr sells the material of the histologist and will realize that comment or helpful advice can hardly be expected over the range of specialties including histopathology, histochemistry, bacteriology, entomology, and botany. It is essentially a list of methods, and many of them are given without reference; if these are all due to Mr. Gurr one can but be amazed. The first edition, according to the wrapper, was a masterpiece of clarity, and this edition is said to have an exhaustive and careful index. Sampling of the latter reveals Van Gieson wrongly spelt and without a reference to page 122; stomach referred to p. 172 in mistake for 173 and not correlated with gastric mucosa for which three other page references are given. Ehrlich on p. 131 should be Ehrich, and terpineol is wrongly spelt on p. 122. There is no mention of Steedman's excellent ester-wax technique.

This as a source book does not compare with Peter Gray's meticulous and voluminous *The Microtomist's Formulary and Guide*. As a book for the learner or the thinker it lacks the verity and ring of experience that we meet in the little books written by men on the job. Whatever a reviewer may say it will be bought, for craftsmen, like good wives, have unquenchable curiosity and their own phoenix of optimism. Moreover, every good technician tries out a new method at least once a year, and there are sure to be some in this volume that he has never tried before.

ALAN LENDRUM.

The Pathogenesis of Coronary Occlusion. By A. D. Morgan; Foreword by John B. Duguid. (Pp. 171; 179 figures. 42s.) Oxford: Blackwell Scientific Publications. 1956.

This is a readable book with a pleasant historical survey of the conflict and confusion that bedevil this subject. Even the most modest writers of to-day would do well to remember how Virchow's frown could rusticate the hypothesis of a Rokitansky. The rehabilitation of the latter by Professor Duguid, who contributes a foreword to the volume, is guardedly and then freely accepted by the author. Indeed, although the other views are given a judicial assessment and their probable participation frankly recognized, the thrombotic element in the occlusive process is expounded with objectivity and clarity. The illustrations, many of which are excellent, contribute significantly to this bit of the argument.

Running through the book is an apparent but not emphasized acceptance of the now almost fashionable view that arterial endothelium is more permeable than we had once imagined, and, as Anitschkow showed, that the internal elastic lamina acts as a fairly effective barrier to the larger molecules that seep in. He illustrates in an entertaining but rather unrealistic coloured frontispiece the escape of lipid into the media once the internal elastic lamina is broken. Perhaps the mucinous degeneration of the intima, which he suggests as a possible localizing factor in the atheromatous process, may itself be the result of the sentrance of some substance from the blood stream, a entrance of some substance from the blood stream, a in the amount passing into the wall.

When the author comes to the conclusions one notes with disappointment that there is no loss of fluency in the writing and it becomes clear that he is coming down on all sides of this curious fence. His forensic display has the merit of arranging the evidence, of poising view against view, and generally of showing where reasonable men could agree, although he swerves past the Whitechapel paradox, that apparently odd retrogression of atheroma at the London Hospital during the 50 years in which myocardial infarction has been so rapidly increasing. Occlusion of the coronaries remains almost as mysterious as it was, but those who are intrigued will be grateful to Dr. Morgan. Carmine on p. 97 should, of course, be azocarmine, a very different chemical substance.

A. C. LENDRUM.

Ciba Foundation Colloquia on Ageing, Vol. 3. Methodology of the Study of Ageing. Edited by G. E. W. Wolstenholme and Miss Cecilia M. O'Connor. (Pp. x+202; 46 figures. 32s. 6d.) London: J. and A. Churchill. 1957.

This, the third of the colloquia on ageing organized by the Ciba Foundation, was designed to explore the avenues of approach available for the study of human gerontology. Thirteen of the 28 participants are responsible for the communications, most of which are followed by discussions, informal, lively, and most ably reported. The themes are varied and important.

It is salutary for the medical reader to learn about ageing in creatures other than man, for the pattern so often is different. Fertility, for instance, increases rather than declines with age in snakes and in the North Sea haddock (Bourlière). Although the theory originally put forward by Rubner (1928), that there is a direct relationship between the rate of metabolism and lifespan, has often been challenged it would appear to be true for invertebrates as well as, generally speaking, for both poikilothermal and homoiothermal vertebrates. Sticklebacks, for instance, pay the price for living in France with a life shortened to 14 or 18 months: they live longer in northern climates. Likewise pilchards survive longer in the English Channel than off Saint Jean-de-Luz. In the mammalian world it is significant that those animals with poor tempera-

ture regulation and a normally low rate of metabolism tend to have a longer lifespan than others of like size.

For the pathologist, the unequal distribution of atherosclerosis in animals is of interest, and Bourlière believes that its role as a degenerative process in ageing phenomena has been greatly overestimated. The question of premature ageing in primitive communities clearly requires thorough investigation before it is too late. Professor T. Gillman's observations on nutritional siderosis in certain African communities provide an important lesson in this connexion.

Of the many fascinating subjects dealt with in this colloquium, Dr. F. J. Kallmann's studies on elderly twins are most revealing and show the overriding importance of inheritance. Deficiency states, both physical and mental, may, it seems, be gene-determined. The schizoid personality structure, he claims (1952), is that of a heterozygous carrier of the schizophrenic genotype with an inadequate degree of general constitutional resistance. But, if this makes depressing reading for the psychiatrist who has to treat schizophrenics, all readers who are getting on in years will be cheered to learn that mental fatigue, up to the age of 60 at any rate, may, contrary to expectations, actually become less with age.

W. H. McMENEMEY.

Dextran and its Use in Colloidal Infusion Solutions. By Anders Grönwall. (Pp. 156; 28 figures. 20s.) Oxford: Blackwell Scientific Publications; New York: Academic Press. 1957.

This little book, by the leader of the team which introduced dextran into clinical practice, is an essential volume for the bookshelf of everybody who is concerned with the theory or practice of blood transfusion. The whole history of dextran is summarized in 156 pages and eight chapters supported by 446 references. Starting with a general discussion of the problem of colloidal infusion solutions, the reader is led through the mysteries of the production and chemistry of undegraded and degraded dextrans and the methods by which the dextran molecule can be adapted to produce desired therapeutic effects: this is written clearly and in such a way that the reader is never allowed to forget that all this work is devoted to producing a safe and efficient therapeutic agent. Then a discussion on the fate of dextran in the body leads on naturally to a discussion on its serology, while the last 56 pages deal with clinical investigation.

This is quite obviously a book written by one man, and bears the mark of his personality and philosophy. Fortunately this makes it extremely agreeable to read; he is not afraid to lead on by easy stages into complex problems of biosynthesis, of light-scattering and viscosity in relation to molecular size and shape; and no more is he ashamed to utter, once more, the most elementary truths, provided he believes that they are needed; the result is that he has made a book which is an essential work of reference for the transfusionist.

I believe that many students and registrars could profit from it, not as a text on dextran, but as an exposition of the complex problems of modern pharmaceutics, wherein the practitioner, biologist, chemist, atomic physicist, and others unite in a single aim: its subtitle could be "The Integration of Experiments."

This book has been translated from the Swedish by Rudolph C. Ellsworth, M.S.; he is to be congratulated on a remarkable achievement, for I can find no internal evidence that the book has been translated.

GEORGE DISCOMBE.

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THE NATURE, DETERMINATION, AND CLINICAL IMPORTANCE OF BLOOD IODINE: A REVIEW

BY

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Normal and Abnormal Constituents of Blood Iodine

In normal conditions, serum (or plasma) iodine is largely composed of thyroxine and iodide with small quantities of thyroactive iodothyronines, such as 3:5:3′-tri-iodothyronine and 3:3′-di-iodothyronine (Gross and Pitt-Rivers, 1952; Roche, Lissitzky, and Michel, 1952a and b; Roche, Michel, Nunez, and Wolf, 1956). Another relatively less active compound, 3:3′:5′-tri-iodothyronine, is present in thyroid gland hydrolysates (Roche *et al.*, 1955) and may be present in plasma in quantities too small to detect, since it is not broken down by the thyroid (Roche *et al.*, 1956).

Mono- and di-iodotyrosine, known to be present in thyroid hydrolysates (Harington and Randall, 1929; Fink, Dent, and Fink, 1947), are not found in normal sera, being deiodinated by an enzyme present in the thyroid gland (Roche, Michel, Michel, and Lissitzky, 1952). In certain types of familial goitre, however, mono- and di-iodotyrosine are present in serum as a result of the absence of the thyroid deiodinating enzyme (Stanbury, Kassenaar, Meijer, and Terpstra, 1955; Querido, Stanbury, Kassenaar, and Meijer, 1956; Stanbury, Meijer, and Kassenaar, 1956).

Thyroxine and, to a lesser extent, 3:5:3′-tri-iodothyronine have been shown to be bound to a specific plasma protein, intermediate in properties between α_1 and α_2 globulins (Trevorrow, 1939; Taurog and Chaikoff, 1948; Gordon, Gross, O'Connor, and Pitt-Rivers, 1952; Deiss, Albright, and Larson, 1953; Petermann, Robbins, and Hamilton, 1954; Robbins, Petermann, and Rall, 1955). No data are available for mono-iodotyrosine or for other iodothyronines. The iodine fraction, which is precipitated with protein, mainly consists of thyroid hormone and is termed "protein-bound iodine (P.B.I.)."

Red cells normally contain iodide but no iodinated amino-acids (Joliot, Courrier, Horeau, and Süe, 1944; Ingbar, Freinkel, Hoeprich, and Athens, 1954; Tata, 1954, quoted by Michel, 1956). However, red cell stroma adsorbs 3:5:3′-tri-iodothyronine (Hamolsky, 1955; Crispell, Kahana, and Hyer, 1956; Crispell and Coleman, 1956), so small amounts of this amino-acid might be found in erythrocytes if its plasma concentration was high.

Iodine-containing proteins may sometimes be detected in serum. Thyroglobulin is liberated into the blood as a result of thyroid surgery (Lerman, 1940) and 48 hours after the destruction of the thyroid by radio-iodine (Feller, Chaikoff, Taurog, and Jones, 1949; Tong, Taurog, and Chaikoff, 1952; Robbins, 1954; Robbins *et al.*, 1954). Dobyns and Hirsch (1956) found that a substance, subsequently identified as thyroglobulin by isoelectric precipitation, electrophoresis, and ultracentrifugation (Dobyns, personal communication, 1956), was secreted into the cervical lymphatics of dogs in response to thyrotropic hormone. The dogs were given 1.4–3 mc. of ^{131}I, thus enabling the labelled thyroglobulin to be detected. Most of the experiments were performed within 24 hours of administering ^{131}I, but the fact that little or no thyroglobulin was present in the cervical lymph before giving thyrotropic hormone makes it unlikely that radiation damage to the thyroid was in any way responsible for their results. Other types of iodine-containing protein have been found in some samples of sheep and calf blood (Taurog, Wheat, and Chaikoff, 1956) and in the sera of patients suffering from carcinoma of the thyroid treated with large doses of iodine, the latter proteins resembling albumin (Larson, Deiss, and Albright, 1954; Robbins, Rall, and Rawson, 1955; Dingledine, Pitt-Rivers, and Stanbury, 1955; Tata, Rall, and Rawson, 1956).

Determination of Blood Iodine

Since plasma contains all, or nearly all, the blood hormonal iodine in loose combination with

protein, a method of estimating the concentration of P.B.I. in plasma (or serum) would provide a measure of the amount of circulating thyroid hormone.

Such determinations are not very specific, since thyroxine can probably not be distinguished from other iodothyronines, although full data are not yet available. Iodine present in serum as thyroglobulin or in an abnormal serum albumin component would also be estimated as P.B.I., though it is not yet certain how accurately the iodine content of these proteins would be estimated by ordinary methods of P.B.I. determination. A number of artefacts may also interfere with the determination, as described later in connexion with the interpretation of individual analyses.

Methods of estimating iodine in organic combination can be classified into two main groups. In the first group incineration with alkali is used to destroy organic material, inorganic iodide being eluted from the resulting ash, while in the second group organic material is ashed by acid oxidizing agents, such as chromic and perchloric acids, or permanganate, in solution. The iodate so formed is then reduced to iodide and separated from the reaction mixture by distillation. In each case the inorganic iodide is usually estimated by the catalytic method originally described by Sandell and Kolthoff (1937), but some workers use a volumetric method (e.g., Riggs and Man, 1940).

The determination of serum inorganic iodide has not been used to a great extent in the assessment of thyroid function, because, although iodide is a normal blood constituent, its concentration is affected by the iodide intake.

Acid Distillation Methods.—The great technical problem in acid distillation methods is the distillation process itself. If a method of this type is to be of any practical use submicrogram quantities of iodine must be distilled from the digestion fluid and collected without loss, or at any rate with a constant loss. This has necessitated the construction of complicated, specially designed stills (Chaney, 1940; Taurog and Chaikoff, 1946; Barker, 1948). Attempts, such as that of Sobel and Sapsin (1952), to simplify the apparatus usually result in less reliable results. The method of Chaney (1940) gives satisfactorily reproducible results according to Sobel and Sapsin (1952) and Blackburn and Power (1955), but Chaney himself reported a recovery of only 84% when the sample contained 0.05 μg. of iodine, while 93% and 100% recoveries respectively were obtained with samples containing 0.5 μg. and 50 μg. of iodine. The methods of Taurog and Chaikoff (1946) and Barker (1948) are subject to losses of 10 to 15% during the distillation, while acid distillation methods, other than that of Chaney (1940), do not give very satisfactory results (see Table I). Since each distillation requires individual supervision, the number of simultaneous determinations which can be carried out is limited. The use of specially constructed glass apparatus is also a disadvantage. This explains why acid distillation methods have not usually been found suitable for use in a routine laboratory.

TABLE I

RELIABILITY OF SOME PUBLISHED METHODS OF DETERMINING SERUM PROTEIN-BOUND IODINE
(Acland, 1957, with additional data)

Author(s)	Method	S.D. or Coefficient of Variation for Single Estimation	Mean Difference between Duplicates (μg. I/100 ml.)
Acid Distillation			
Gottschalk and Riggs (1952)	Man *et al.* (1942)	±0·46 μg./100 ml.	—
Stanbury *et al.* (1954)	Barker (1948) modified	±0·39 μg./100 ml.	0·42
Sobel and Sapsin (1952)	Chaney (1940)	±4·0%*	0·15
	Sobel and Sapsin (1952)	±7·7%*	0·34
Blackburn and Power (1955)	Chaney (1940) modified	(*a*) ±0·265 μg. I/100 ml. (mean P.B.I. concn. 4·81 μg./100 ml.)	—
		(*b*) ±0·487 μg. I/100 ml. (mean P.B.I. concn. 16·2 μg./100 ml.)	—
Friis (1955) ..	Barker (1948)	±0·44 μg./100 ml.	—
Alkaline Ashing			
Caraway (1952)	Barker *et al.* (1951)	—	0·5
Lamberg *et al.* (1956)	Barker *et al.* (1951)	±0·79 μg./100 ml.†	—
Skanse and Hedenskog (1955)	Barker *et al.* (1951) modified	±0·22 μg./100 ml.	—
Thompson *et al.* (1956)	Barker *et al.* (1951) modified	±5·7% ‡	—
Acland (1957) ..	Barker *et al.* (1951) modified	±0·17 μg./100 ml.	—

* Calculated from the 95% range for the mean of duplicate estimations as given by the authors (±5·6% and ±10·9% respectively).
† Calculated from the standard error for the mean of a duplicate as given by the authors (±0·56).
‡ Calculated from the coefficient of variation for the mean of triplicate determinations as given by the authors (±3·3%).

Methods have been described which use acid digestion to convert organically bound iodine to an inorganic form but omit the distillation step, e.g., that of O'Neal and Simms (1953). Iodate is determined directly on the diluted digest. Although such techniques are simple to use (Reals, Townsend, and Danielson, 1956), the digestion process still requires supervision. O'Neal and Simms (1953) obtained variable results from day to day and recommended the construction of new calibration curves with each batch of determina-

tions. The standard deviation of duplicate analyses from their mean was 0.33 μg./100 ml. The reproducibility attained is thus comparable to that of acid distillation methods as a whole, but is not as good as that of the best techniques, whether based on acid distillation or on alkaline ashing (see Table I).

Critical Examination of Alkaline Ashing Methods.—There are four stages in the estimation of serum P.B.I. by alkaline incineration: (1) Precipitation of serum proteins, (2) ashing, (3) elution of iodide from the ash, and (4) estimation of the released iodide.

In the protein precipitation stage, errors may arise both from incomplete precipitation of P.B.I. and from co-precipitation with protein of iodine-containing compounds such as di-iodotyrosine, which do not normally form part of the P.B.I. According to Tong *et al.* (1954), only 90% of ^{131}I-labelled thyroxine added to serum is recovered in a zinc hydroxide precipitate, while 95% is recovered in a trichloroacetic acid precipitate. No data are available for mono-iodotyrosine. Subsequent investigators have concluded, however, that losses in this stage of the analysis should not be more than 5% with zinc hydroxide, provided that the concentration of zinc is adequate (Skanse and Hedenskog, 1955; Acland, 1957). It may be that the high concentration of thyroxine (10–20 μg./ml.) used by Tong *et al.* (1954) overloaded the thyroxine-binding mechanism.

Appreciable amounts of inorganic iodide (2–3%) may remain in a washed trichloroacetic acid precipitate (Taurog *et al.*, 1956), perhaps owing to the iodination of unsaturated lipids. In the case of zinc hydroxide precipitates also, therapy with potassium iodide, resulting in raised serum iodide levels, may give rise to spuriously high analytical results. Slade (1956) used a column of ion-exchange resin to separate inorganic from organic iodine in plasma. The plasma sample is run through the column, which retains the inorganic iodide, while the effluent contains the organic iodine. This method, while valuable for radio-iodine studies, is not a substitute for protein precipitation in chemical determinations, since the resin column was found to be a source of iodine contamination. Resin column purification is not worth while adopting as a routine in P.B.I. determinations, but it may be a useful alternative to repeated washing of the protein precipitate if the plasma inorganic iodide concentration is very high.

Losses of iodine may occur during ashing unless the temperature is kept below 620° (Acland, 1957). Inconsistent results have been obtained when trichloroacetic acid precipitates are ashed below 650° whereas above this temperature recoveries become low (Acland, 1957).

Grossmann and Grossmann (1955) drew attention to the possibility of losses in acid solution due to the conversion of iodide to iodine at the elution stage. The eluents used by Barker, Humphrey, and Soley (1951) and Brown, Reingold, and Samson (1953) have been found to give low recoveries (Acland, 1957) and the same results would be expected whenever elution takes place in acid conditions. The amount of acid added to the ash in the procedure recommended by Grossmann and Grossmann (1955) is enough to break up the residue but not enough to make the resultant suspension acid, and these authors' elution technique was found to give satisfactory results (Acland, 1957). Adsorption on the carbonized residue probably accounts for the losses of iodine occurring with acid eluents, since acidification *after* separation of the supernatant from the residue, as in the method of Grossmann and Grossmann (1955), does not result in a low recovery (Acland, 1957).

The estimation of iodide by its catalytic effect on the reaction between ceric salts and arsenious acid (Sandell and Kolthoff, 1937) depends upon the fact that the reduction of the ceric ion is normally a first order reaction, the rate-limiting process being the reaction between ceric ions and iodide (Glasstone, 1948). By a kinetic approach similar to that of Caraway (1952) it has been shown (Acland, 1957) that in the conditions of the analysis a linear relation should theoretically exist between the logarithm of the concentration of unchanged ceric ion and the amount of iodide present. Provided that ceric salts obey Beer's law, a straight line calibration curve should result if extinction is plotted against iodide concentration on semilogarithmic graph paper. Ceric salts in solution obey Beer's law only if the spectrophotometer used has an adequate monochromator, giving a performance equal to that of the Beckmann DU (Thompson, Klugerman, and Truemper, 1956) or of the "unicam" SP600 (Acland, 1957). With satisfactory instrumentation, a theoretical straight line calibration curve can be obtained (Acland, 1957) in the presence of chloride and sulphate, the former of which enhances the catalytic effect of iodide (Barker, 1948) while the latter retards it (Klein, 1954).

Several workers (e.g., Grossmann and Grossmann, 1955; Meyer, Dickenman, White, and Zak, 1955; Fischl, 1956) have reported methods of poisoning the ceric sulphate-arsenious acid reaction, so that photometer readings may be made at any time after the reaction has stopped. This technique is claimed to be more accurate than that whereby readings are made at a fixed time after starting the reaction, while the optical density is still decreasing. On the face of it, this appears an attractive procedure. However, the colour obtained is not always stable, although Fischl (1956) claims that in the case of the brucine acetate-cerium complex full intensity can be developed by heating to 95–100° for 5 min. Usually there is no difficulty in timing the extinction reading to within 1 or 2 sec. and constancy of delivery time can be ensured by using the same pipette throughout to deliver the ceric ammonium sulphate solution (Acland, 1957).

Ultramicromethods. — In the case of most methods of P.B.I. determination, a venepuncture is necessary, since 0.5–1.0 ml. samples of serum are required. Fischl (1956), however, has described a technique which uses only 0.02–0.1 ml. of serum and would therefore be suitable for finger-prick blood. The necessary increase in sensitivity was claimed to result from the use of brucine acetate to stop the reaction between ceric and arsenite ions. The colour of the brucine acetate-cerium complex is much more intense than that of the ceric ion. In comparison with other methods, therefore, the light absorption can be measured at a higher wavelength (540 mμ instead of about 420 mμ), while the slope of the calibration curve relating optical density to iodide concentration is much steeper. Fischl (1956) claims that an increased accuracy results from the greater sensitivity. This claim applies, of course, only to spectrophotometric errors in the determination of ceric ion concentration. Errors due to contamination of the reagents with iodide remain proportionately the same, except in the case of the ceric ammonium sulphate and arsenious acid solutions, where they are about five times greater. The relative effect of pipetting errors involving the ceric ammonium sulphate solution is also increased five times. Errors in the digestion (or incineration) stage will not be affected, except possibly adversely, as a result of the smaller quantities of starting material. Fischl (1956) plots photometer readings (in logarithmic units) against iodine concentration to obtain a straight line calibration curve. As stated previously, this procedure has no theoretical justification since a straight line relation should exist between the logarithm of the optical density and the iodide concentration. It is possible that the use of a Klett-Summerson filter photometer may have prevented the brucine acetate-cerium complex from obeying Beer's law, since Thompson *et al.* (1956) were unable to obtain satisfactory results from this instrument with ceric salts in the absence of brucine.

Fischl (1956) has adapted his method both to a chloric acid digestion without distillation, similar to that of O'Neal and Simms (1953), and to an alkaline ashing technique, each resulting in different calibration curves. The acid digestion gives a higher blank and apparently a wider variation between duplicates, while alkaline ashing gives a steeper fall of optical density as the iodine concentration rises. However, the ashing was carried out at 650°, although only for 12 to 16 min., and the ash was acidified with sulphuric acid, so losses of iodine are very likely to occur for the reasons already given.

Fischl (1956) quotes a "1.5–3.0% deviation," obtained with triplicate determinations on samples of serum from hypothyroid, euthyroid, and hyperthyroid patients. A "range of error" of 5 to 8% was observed using smaller quantities of serum and reagents in a Klett micro tube. No figures are given for the recovery of thyroxine, either alone or added to serum.

Another attempt to develop an ultramicrotechnique has been reported by Sanz, Brechbühler, and Green (1956). They followed in most respects the procedure of Barker *et al.* (1951), scaled down to correspond to a serum sample of 0.05 ml. However, a Beckmann DU spectrophotometer was used and the light absorption of the ceric ion was measured at its absorption maximum, 317 mμ instead of at the higher wavelengths normally used. As a result, while the concentration of iodide in the final reaction mixture remained the same as in the macro method, that of the ceric ion was reduced ten times. It seems very likely that in these conditions the concentration of the ceric ion would be too small for its reduction to follow first-order kinetics. Failure of this relation to hold at high iodide and low ceric ion concentrations has been observed (Thompson *et al.*, 1956; Acland, 1957). Sanz *et al.* (1956) observed that the transmittance at 317 mμ showed a linear relation both with the reaction time and with the iodide concentration. Theoretically, the logarithm of the extinction and not the transmittance should show a linear relation with the last two variables (Barker *et al.*, 1951;

Acland, 1957). It is possible that the failure of the reaction in the experimental conditions to follow first order kinetics was compensated purely fortuitously by what is in effect a double antilog transformation.

Sanz *et al.* (1956) claim a precision of better than ±1% and report a mean recovery of 99.7% for thyroxine added to seven serum samples. They determined serum P.B.I. concentrations in 12 normal subjects and found them to lie between 3.52 and 5.59 μg./100 ml. (mean, 4.81). They comment that in Swiss subjects P.B.I. values are low owing to the lack of iodine in the water, but they do not report any clinical or other evidence relating to the thyroid function of their subjects. Protein-bound iodine determination by the alkaline incineration method of Barker *et al.* (1951) has been found to be subject to variable losses, probably during the iodide elevation stage after ashing (Brown *et al.*, 1953 ; Acland, 1957). Conversion to the ultramicro scale would normally be expected to enhance rather than eliminate such losses. Sanz *et al.* (1956) also claim good agreement between the results of their technique and those of the unmodified Barker method when applied to the same serum specimen.

Further confirmatory work appears to be necessary before pronouncing judgment on these ultramicromethods for P.B.I. determination.

Conclusion.—As shown by the representative data quoted in Table I, alkaline incineration methods are capable of giving more reproducible results than acid distillation methods. Acland (1957) reported a mean recovery of thyroxine added to serum of 93% (range 89–97%) using an alkaline incineration method. Acid distillation methods usually have recoveries in the range 85–90% (Taurog and Chaikoff, 1946 ; Barker, 1948 ; van Zyl, 1953). Alkaline incineration methods cannot, however, be used satisfactorily unless there is a large excess of protein in the starting material (Acland, 1957). Van Zyl (1953) claims, on the other hand, that acid distillation methods enable small quantities of inorganic and organic iodide to be determined accurately in starting materials containing little protein, the important factor being a careful control of the concentration of the reagents used. Another disadvantage of alkaline ashing methods is that trichloroacetic acid precipitation gives inconsistent results when combined with this technique (Acland, 1957). Trichloroacetic acid precipitation can, however, be used satisfactorily in conjunction with acid distillation methods (Sobel and Sapsin, 1952). It would obviously be preferable to use trichloroacetic acid if possible in view of the co-precipitation with the P.B.I. of physiologically inactive substances, such as di-iodotyrosine, when zinc hydroxide reagents are used.

The Chemical Fractionation of Serum Iodine

The suggestion has often been put forward (e.g., Blomfield, Jones, Macgregor, Miller, and Wayne, 1951 ; Starr and Liebhold-Schueck, 1953) that different dysthyroid states might be characterized not only by quantitative changes in the amount of thyroactive substances secreted by the thyroid, but also by changes in its composition. Both the production by the thyroid of abnormal compounds and an alteration in the relative proportions of its normal secretory products have been postulated. It has consequently appeared important to many investigators to develop fractionation methods which would enable each component of serum iodine to be estimated separately.

The *n*-butanol extraction methods of Leland and Foster (1932) and Blau (1933) are often used. Plasma is extracted with *n*-butanol in acid conditions and the extract washed with 4N-NaOH in 5% (w/v) Na_2CO_3 (Blau's reagent). Thyroxine stays in the *n*-butanol layer, while di-iodotyrosine and iodide pass into the alkaline washings. This technique is insufficiently specific and would probably not distinguish between different iodothyronines (Roche and Michel, 1955). Moreover, extraction of iodine from blood is not complete and some thyroxine is broken down during the separation.

It seems hardly justifiable, therefore, to use the serum butanol extractable iodine (B.E.I.) for clinical purposes, as recommended by Man, Kydd, and Peters (1951). Man, Bondy, Weeks, and Peters (1954) give a normal range for serum B.E.I. of 3.2 to 6.4 μg./100 ml. This is, as might be expected, lower than the range accepted for serum P.B.I., 4–8 μg./100 ml. (Roche and Michel, 1955).

The only promising fractionation technique for serum iodine is that described by Bowden, Maclagan, and Wilkinson (1955). It gives semi-quantitative results but is highly specific and is probably capable of further development to increase its accuracy. One-dimensional chromatograms of *n*-butanol extracts containing iodinated compounds are treated with a ceric sulphate-arsenious acid reagent and the degree of decolorization of the yellow ceric ion measured spectrophotometrically in the ultra-violet at intervals along the paper strip. Over the range 0.1–0.3 μg. of thyroxine (or tri-iodothyronine)

there is, in the conditions used by Bowden *et al.* (1955), a linear relation between the galvanometer reading and the amount of amino-acid present. The range over which this relation can be observed is limited by the relative dimensions of the spot and the photocell.

Precautions must be taken to guard against chromatographic artefacts. The chromatographic solvent system, *n*-butanol-dioxane-2*N* ammonia (4:1:5 by vol.), recommended by Bowden *et al.* (1955), causes degradation of ^{131}I-labelled thyroxine in the tri-iodothyronine and iodide areas unless the dioxane is redistilled to free it from peroxides (Owen, McKenzie, and Orvis, 1956). Moreover, if acidic solvents are used in extraction or chromatography, exchange can occur between iodide and di-iodotyrosine, while free iodine is formed from iodide, which can then iodinate, e.g., unsaturated lipids (Gross and Leblond, 1951; Acland, 1952).

The method of Bowden *et al.* (1955) requires serum iodine to be concentrated before chromatography. This is best done by extraction with *n*-butanol and subsequent concentration of the extract. This means, however, that the results of the determination will be affected by the incomplete extraction of thyroxine mentioned above. It is, further, not known whether the *n*-butanol-soluble, albumin-like serum iodine component of Robbins, Rall, and Rawson (1955) is detectable on chromatograms by the ceric sulphate-arsenious acid reagent. Two main lines of improvement seem possible. First, an extraction method might be developed which would extract all the thyroxine-like iodine. This would remove one of the main sources of error. Secondly, a refinement of the spectrophotometric technique might increase the degree of correspondence between the galvanometer reading and the amount of iodinated compound present. It might be better to apply the extract to the paper along a starting line instead of to a point and to use a scanner fitted with a slit, similar to the commercial models designed for use with electrophoretograms. A "profile" curve of the chromatogram strip could then be constructed as for serum proteins, by plotting the galvanometer deflection against the distance along the chromatogram. The scanner could be calibrated for the serum iodine components, provided that the area under a segment of the curve were proportional to the amount of iodinated compound contained in the corresponding spot.

Ideally, it might be hoped that a routine method for fractionating serum iodine could be based on the technique of Tong *et al.* (1952), in which untreated serum is chromatographed directly. However, the amounts of thyroxine iodine so added are about a hundred times too small to be estimated by the method of Bowden *et al.* (1955) nor can they be readily detected on the paper by radiochemical techniques unless the subject has received a dose of radio-iodine in the millicurie range. Doses of this size are given only for therapeutic purposes. Even with the column chromatography technique of Gross and Pitt-Rivers (1952), which, incidentally, also uses *n*-butanol extracts as starting material, radioactivity measurements from patients given tracer doses of a few microcuries present considerable difficulties.

At the present time the problems posed by serum iodine strain both radio-isotope and chemical techniques to their limit. The ultimate goal is a determination of the specific activity of each fraction of serum iodine isolated chromatographically. Only in this way can the function of the thyroid be adequately assessed. Rough specific activity measurements are in theory possible at the moment using rather large doses of radio-iodine and rather large serum samples. It is probable that with the development of scintillation counting and improvement in the chemical determination of Bowden *et al.* (1955) sufficient accuracy will be attained to make such estimations worth while. However, it would probably not be practicable to carry out this investigation as a routine on all patients suffering from thyroid disease, since the measurements are likely to remain too laborious to permit a large number of determinations to be made simultaneously.

Serum P.B.I. and Assessment of Thyroid Function

Thyroid function can be assessed at three different physiological levels. First, the rate of hormone production can be estimated by means of radio-iodine tests. Secondly, the concentration of circulating thyroid hormone with which the tissues are in contact can be estimated by means of serum P.B.I. determinations. Thirdly, the reaction of the tissues themselves to the thyroid can be assessed clinically and by means of laboratory tests such as measurements of the basal metabolic rate or the blood cholesterol. In about 90% of cases of hyper- and hypothyroidism it is found that the results of all tests are in agreement.

The level of serum P.B.I. represents a metabolic equilibrium between synthesis and breakdown of thyroid hormone superimposed upon the physicochemical equilibrium between thyroid hormone in the serum, where it is bound to protein, and in the tissues.

In most cases the serum P.B.I. concentration in μg./l. (B) is a good measure of the rate of hormone supply in μg./day (H) and is related to it by the rather simple equation (Riggs, 1952) $B^2 = 34.1\ H$. This equation was derived by a combination of theoretical and empirical calculations and was found to be obeyed rather closely in studies on hypothyroid and euthyroid subjects who had been given thyroid by mouth. The relation assumes a constant clearance rate of hormone from the extrathyroidal body compartment (measured in litres cleared/hr./μg. hormonal I in the compartment) and a constant volume of distribution of hormonal iodine relative to the plasma hormonal iodine concentration. These assumptions are not necessarily correct in all situations. An increased clearance rate may occur in adolescence or exposure to cold, thereby leading to an underestimate of hormone production, while a decreased clearance rate may occur in old age leading to a corresponding overestimate. On the other hand, where the concentration of plasma protein is low, as in nephrosis, the volume of distribution of organic iodine in the extrathyroidal compartment relative to the serum P.B.I. concentration may be increased. This could result from an increase in the proportion of unbound thyroxine in plasma when the concentration of thyroxine-binding protein is lowered. In this case, the serum P.B.I. will underestimate the rate of hormone production.

The range within which determinations of serum P.B.I. fall in the case of euthyroid individuals depends on the method used. It is probable that, with the best techniques, most normal values will be found to lie between 4 and 8 μg. P.B.I./100 ml. serum, as stated by Roche and Michel (1955). Values below or above these limits are usually obtained in thyroid diseases. There are reasons, however, for suspecting that the upper limit of normal should be set higher than 8 μg. P.B.I./100 ml. In the case of a method of P.B.I. determination such as that of Barker *et al.* (1951), which is probably subject to systematic and sporadic losses (Acland, 1957), both limits of the euthyroid range may be lower, certain sera from hyperthyroid patients may appear in the euthyroid range, and some sera from euthyroid patients may appear in the hypothyroid range. This is quite apart from the overlap which would be expected between the serum P.B.I. concentration ranges in normal and pathological conditions. The results of several groups of workers reporting normal ranges of 3 to 7.5 or 8 μg. I/100 ml. may be open to criticism on these lines (e.g., Walser, Ludwig, and Ortelli, 1954; Sunderman and Sunderman, 1954; Lamberg, Wahlberg, and Forsius, 1956).

The interpretation of a serum P.B.I. determination in a patient is not always straightforward, since, in the first place, the analysis itself is subject to interference by artefacts and, secondly, P.B.I. concentrations outside the normal range are found in some physiological and pathological conditions in which thyroid disease is absent.

Artefactual elevation of serum P.B.I. results from iodide administration in the form of Lugol's iodine, cough mixtures containing potassium iodide, vitamin capsules, etc. This effect persists for a period of days. Serum P.B.I. values remain raised for months or even years after the administration of contrast media for radiography. Raised P.B.I. values are recorded in hyperthyroidism of all types, including cases of acute diffuse (granulomatous) thyroiditis (Owen and McConahey, 1956) and Hashimoto's thyroiditis in the acute phase. Ingestion of a daily dose equivalent to at least 0.2–0.6 g. of thyroid extract is necessary to raise the serum P.B.I. concentration of euthyroid subjects above normal limits (Chamovitz, Sleisenger, and Freedberg, 1951; Danowski, Huff, Tarail, Wirth, Peters, Mateer, and Garver, 1952).

In physiological conditions P.B.I. levels, high in comparison with the normal adult range, are found in children (Durham, Cooke, Lancaster, and Man, 1954; De Pascale, 1956). Values above 10 μg. of P.B.I./100 ml. of serum may be observed in the first month of life, a gradual decrease occurring with increasing age until values fall within the normal adult range at about 7 years of age. At puberty the serum P.B.I. level rises slightly before returning to its normal adult level. High values are also obtained in normal pregnancy. By the sixteenth week the serum P.B.I. concentration often rises above the normal limits for adults without evidence of thyrotoxicosis (Heinemann, Johnson, and Man, 1948; Man, Heinemann, Johnson, Leary, and Peters, 1951; Lum and Man, 1955). Failure of the serum P.B.I. to rise during pregnancy is regarded by Lum and Man (1955) as a good indication for thyroid medication to prevent abortion.

A raised serum P.B.I. has been reported in the early stages of acute hepatitis (Kydd and Man, 1951). Cattaneo, De Simoni, and Fantoli (1955) found raised serum P.B.I. levels in patients suffering from pulmonary tuberculosis and observed a further rise after surgical intervention. This last finding is surprising since Engstrom and Markardt (1955) could not detect any effect of surgical stress on serum P.B.I. in patients who were not suffering

from a concurrent infection. Bonati, Salvi, Rancati, and Pedrelli (1955) have found that a temporary rise in serum P.B.I. follows insulin coma therapy in psychiatric patients.

Artefactual depression of serum P.B.I. may result from the administration of mercurial diuretics since mercury salts interfere with the distillation of iodine and with the chemical determination of iodides by the catalytic method. A 24-hr. interval between medication and taking the blood sample is usually sufficient to allow for elimination of mercury from the blood stream (Barker, personal communication, 1955).

Lowered serum P.B.I. levels are found in hypothyroidism of all types, including Hashimoto's disease in its later stages, familial goitrous cretinism (McGirr and Hutchison, 1953; Stanbury *et al.*, 1955), and hypothyroidism following subtotal thyroidectomy or radio-iodine therapy. Usually a low serum P.B.^{127}I. is associated with a low serum P.B.^{131}I. and a decreased thyroid uptake of ^{131}I. However, in the case of familial goitrous cretins with low serum ^{127}I the 48-hr. serum P.B.^{131}I. is often raised and the thyroid uptake is above normal two and a half hours after administration of ^{131}I, although the 24-hr. ^{131}I uptake is normal. Patients rendered hypothyroid by surgery may have a raised thyroid uptake and a high 48-hr. serum P.B.^{131}I. level, although the serum P.B.^{127}I. is low (Blom and Terpstra, 1953), and the same may be true of patients rendered hypothyroid by radio-iodine (Blomfield *et al.*, 1951). These anomalous results arise because the surviving thyroid tissue is incorporating iodine more rapidly into the hormone it secretes than the normal thyroid gland does, and this process is reflected in the P.B.^{131}I. level. However, the actual rate of hormone secretion, on which the P.B.^{127}I. level depends, is low since there is less than the normal amount of active tissue present as a result of therapy or of the disease process.

Cortisone administration may cause a fall in serum P.B.^{127}I., together with lowered values for the B.M.R. and ^{131}I tests (Hill, Reiss, Forsham, and Thorn, 1950). Lowered values may also be found in cirrhosis of the liver, sometimes associated with increased thyroid uptake of ^{131}I (Kydd and Man, 1951; Mueller, Brausch, Hirsch, Benua, and Dobyns, 1954). These findings probably result from the low plasma protein level in such cases. Debilitating diseases, such as pneumonia, carcinomatosis, malnutrition, terminal heart failure, haemochromatosis, renal failure, gangrene, and fractured femur, cause a lowering of serum P.B.I. (Engstrom and Markardt, 1955). Bonati *et al.* (1955) find that the serum P.B.I. level falls in psychiatric patients treated for 10 days with chlorpromazine and phenobarbital. Beierwaltes (1956) has reported a lowered serum P.B.I. together with a raised B.M.R. after administration of dinitrophenol.

Normal serum P.B.I. levels are observed in certain metabolic disorders, which finding may be of diagnostic importance. Patients with large goitres may show an increased thyroid ^{131}I uptake as a result of the large size of the gland, but, unless they are thyrotoxic, will not show an elevated serum P.B.^{127}I. An increased basal metabolic rate with a normal serum P.B.I. may be observed in early congestive heart failure, leukaemia, phaeochromocytoma, acromegaly, essential hypertension, and osteitis deformans (see Bréant, 1955; Beierwaltes, 1956). The distinction from thyrotoxicosis thus rendered possible is especially important in cardiac disease. A decreased B.M.R., together with a normal P.B.I., may be found in Addison's disease, hypopituitarism, and anorexia nervosa (see Bréant, 1955). Neither schizophrenia nor affective psychoses appear to be associated with changes in serum P.B.I. concentration (Bonati *et al.*, 1955).

As mentioned above, serum P.B.I. determinations measure only the state of the equilibrium between hormone synthesis and breakdown which is, however, largely determined by the rate of synthesis. Measurements of serum P.B.^{131}I. measure the rate of incorporation of radio-iodine into the circulating thyroid hormone. A combination of the two to yield specific activity measurements would therefore afford a measure of the functional size of the thyroid gland. Although serum P.B.I. determinations by themselves usually correlate well with clinical state (Sunderman and Sunderman, 1954), they are probably most valuable when combined with other tests of thyroid function, including radio-iodine tests and B.M.R. determination, in order to obtain a complete picture of thyroid activity (Fraser, 1956). Methods of determining serum P.B.I. are now available which are sufficiently accurate and reliable for use in a routine chemical pathology laboratory.

Summary

The nature and determination of blood iodine are reviewed critically. The clinical use of serum P.B.I. determinations is discussed. It is concluded that serum P.B.I. determinations can usefully be performed in a routine laboratory.

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APPENDIX

P.B.I. by Alkaline Incineration

The method given below is essentially that previously described (Acland, 1957) with certain modifications introduced as a result of its application to routine estimations (Kilpatrick, 1957). The latter worker found in 20 batches of determinations no significant changes in the calibration curve from batch to batch and no significant difference between duplicate determinations on samples from the same serum analysed in different batches.

Technical Precautions.—Elementary iodine must be excluded from the room where analyses are being carried out and must not be used to sterilize the skin before taking a blood sample. When not in use, glassware is kept immersed in an aqueous solution of an alkaline detergent: "dymex" (Thomas Hedley and Co., Ltd., Newcastle upon Tyne) and "teepol" (British Drug Houses Ltd.) have both been found satisfactory. Immediately before use the glassware is first rinsed thoroughly with tap water, test-tubes being cleaned with a brush, and then washed three times with water purified as described below. Pipettes are dried with acetone on a filter pump, other glassware in a hot air oven at 90°. Containers for blood collection must be kept separate from those used for other laboratory investigations. Scrupulous cleanliness is essential.

Reagents.—All chemicals are AnalaR grade except HCl, H_2SO_4 ("microanalytical reagent," Hopkin and Williams Ltd.), and ceric ammonium sulphate ("laboratory reagent low in other rare earths," British Drug Houses Ltd.). All water is purified by distillation from a metal still and passage through two columns of Amberlite Monobed MB-1 ion-exchange resin. The second resin treatment was given immediately before use.

The KI reference solution contains 130.8 mg. of the desiccator-dried salt/l. ($\equiv$100 μg. of I/ml.). The ceric ammonium sulphate solution contains 19.5 g. of salt in 1 litre of 3.5N H_2SO_4. The use of this lower concentration brings the extinction readings below 1.0. The ceric ammonium sulphate solution may deteriorate after a few months (Kilpatrick, 1957). To guard against this, at the beginning of every week add 0.5 ml. of the ceric ammonium sulphate solution to 5.5 ml. of water, contained in a test-tube, incubate at 37° for about 20 min. to allow the colour change to proceed to completion, and read $E_{1\,cm.}$ at 415 mμ. This procedure has been found to give an extinction reading of 1.4, but other laboratories may obtain different values. The precise value observed is unimportant as long as it remains constant from week to week.

Arsenious acid is prepared by dissolving 3.8 g. of As_2O_3 in 50 ml. of warm N-NaOH. To the cooled solution is added 50 ml. of water and 3.5N H_2SO_4 to 500 ml.

Precipitation of P.B.I.—Pipette two 1 ml. portions of serum into 16 mm. $\times$ 125 mm. "pyrex" tubes. Add to each tube 1 ml. of water, followed by 1 ml. of 100% (w/v) $ZnSO_4.10H_2O$ and 1 ml. of 0.5N NaOH, stirring with a glass rod between each addition. Stand for 3 hr. and separate the precipitates by centrifugation at 2,000 r.p.m. for 10 min. Wash the precipitates three times by resuspension in successive 10 ml. portions of water, centrifuging and discarding the washing. Suspend each washed precipitate in 1 ml. of 4N Na_2CO_3 by stirring with a glass rod and wash the last traces of precipitate into each tube by adding 0.5–1 ml. of water down the rod.

Ashing.—Dry the precipitates overnight at 95–98°. Place the tubes containing dried precipitate in a muffle furnace for three and a half hours at 600°, leaving the furnace door slightly open (about $\frac{1}{4}$ in.) to create a draught. Discard the "pyrex" tubes after they have been used three times.

Estimation of Released Iodine.—Allow the tubes to cool, then add carefully to each residue 1 ml. of 2N HCl and mix with a glass rod. Add to each tube 6 ml. of water and allow the contents of the tube to stand for half an hour. During this time stir the contents of the tubes three times with a glass rod. Transfer the suspensions to conical centrifuge tubes and separate off the carbonized residues by centrifuging for 20 min. at 3,000 r.p.m.

Pipette a 3 ml. sample of each supernatant into a thin-walled 16 mm. $\times$ 150 mm. "pyrex" test-tube. Add to each tube 1 ml. of water, 0.5 ml. of arsenious acid solution, and 1 ml. of 3.5N H_2SO_4 in that order and mix the contents. Place the tubes in a water-bath at 37$\pm$0.1° and after 10 min. add to each tube 0.5 ml. of ceric ammonium sulphate solution at 37°, mix quickly and replace in the bath. The following is a satisfactory method of mixing the contents of the tubes: hold the tube just below its rim in the left hand and flick the bottom of the tube in quick succession with the four fingers of the right hand, repeating this movement about 15 times. Some practice is, however, necessary in order to get good results.

Exactly 12.5 min. after the addition of the ceric ammonium sulphate was begun, measure the extinction of the solution in each tube at 415 mμ. in a 1 cm. cuvette. Filling of the cuvette is started 45 sec. before this time. It is necessary to use a spectrophotometer such as the SP 600 (Unicam Instruments Ltd., Cambridge) equipped with a monochromator. Filter photometers are unsatisfactory. Read the amount of "added iodide," i.e., iodide present in excess of that contaminating the reagents, from a calibration curve, correcting $E_{1\,cm.}$ at 415 mμ. with μg. of "added iodide," whose derivation is described below. Multiply the figure thus obtained by 700/3 to give the result in μg. of I/100 ml. of serum.

It has been found convenient to allow 1 min. between successive additions of ceric ammonium sulphate. This enables six duplicate determinations to

be carried out in one batch. Two such batches can be conveniently dealt with in one two-day period, comprising in all 11 sera estimated in duplicate and two reagent blanks.

Reagent Blanks.—Run two reagent blanks with each day's group of estimations, one reagent blank per batch. Carry out the entire analytical procedure with 1 ml. of water replacing the 1 ml. serum sample. The reagents sometimes take up small amounts of iodine on keeping.

Construction of the Calibration Curve.—To tubes containing 3 ml. of the eluate from a reagent blank, add in place of 1 ml. of water 1 ml. samples of solutions containing respectively 0.02, 0.04, 0.06, 0.08, and 0.10 μg. of I′/ml.; a reagent blank containing no added iodide provides the zero point. Prepare the tubes for each iodide concentration in two batches of 12. Proceed with the addition of arsenious acid and ceric ammonium sulphate as described above. Plot the mean value of $E_{1\ cm.}$ on semilogarithmic graph paper against μg. of added iodide and draw a straight line curve to express the relation between them. Construct a fresh curve whenever a fresh solution of any reagent is made up.

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Addendum

Some advances in the chromatography of iodinated compounds have recently been reported. Maclagan, Bowden, and Wilkinson (1957) have further developed their chromatographic method for determining thyroxine and triiodothyronine in 10 ml. plasma samples, which now includes a modified methanol extraction technique (Brown and Jackson, 1954). The amino-acids are eluted from the paper strip and estimated colorimetrically. No deiodination of thyroxine or triiodothyronine takes place during the preparative procedure. Extreme care is, however, necessary, since this process occurs readily in alkaline solutions. Esterification of iodothyronines in acid *n*-butanol extracts was found to cause the appearance of fast-moving iodine-containing artefacts ($R_F = 0.90-0.95$ in *n*-butanol/dioxane/2N NH_4OH, 4:1:5 by vol.). Renewal of the *n*-butanol/dioxane/NH_4OH solvent every two weeks prevents the formation of peroxide-induced chromatographic artefacts.

Two recently described methods for analysing chromatographically the radioactivity in plasma extracts (Farran, Parker, and Abbatt, 1957; Block, Werner, and Mandl, 1958) are still about five times too insensitive to be used in conjunction with a normal diagnostic dose of ^{131}I (25 μc).

The most sensitive technique yet described for measuring the radioactivity on paper chromatograms is probably that of Taurog, Tong, and Chaikoff (1957), who place sections cut from chromatograms directly into a well-type scintillation counter. It is likely that this technique could be applied profitably to concentrated plasma extracts, but, for maximal reproducibility, the geometrical relation between paper strip and phosphor would have to remain the same in different runs.

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THE DEMONSTRATION OF INTRAVASCULAR HAEMOLYSIS BY MEANS OF SERUM PAPER ELECTROPHORESIS AND A MODIFICATION OF SCHUMM'S REACTION

BY

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In studying the pathogenesis of anaemia associated with many clinical disorders one is often confronted with the problem of whether intravascular haemolysis is taking place. Haemolysis may result either from an intrinsic abnormality of the red cell, rendering it of shorter life span than normal, and this may be of an hereditary or acquired nature, or, alternatively, from the presence of some plasma factor capable of inducing lysis.

Plasma factors producing lysis result from certain types of poisoning, toxic effects of drugs, and many generalized infections. Haemolysis may also occur as a result of renal or hepatic failure and in other metabolic disorders. The exact mechanism of the haemolysis in these conditions, although unknown, is probably also attributable to plasma factors. Despite the wide range of known causative factors many cases of haemolysis remain unclassified, e.g., those which are seen in the reticuloses and other neoplastic states.

In patients manifesting an extreme degree of haemolysis, extracorpuscular haemoglobin can be seen in the serum, which, if in sufficient concentration, is excreted into the urine. More commonly an increased rate of haemolysis is manifest by a raised serum bilirubin level and an increased excretion of urobilinogen in the urine and faeces. Reticulocytosis, if present in such circumstances, reflects a compensatory hyperplasia of the bone marrow, but both these latter diagnostic features may be partially obscured should the rate of bilirubin production be matched by the ability of the liver to excrete it, or should some complicating factor, such as uraemia or chronic infection, simultaneously depress erythropoietic activity. Under these conditions even moderate haemolysis may escape detection by existing routine laboratory procedures, and in the present work two new complementary techniques are described which appear to be more sensitive than other simple procedures, and can demonstrate that haemolysis is occurring or has recently taken place. These tests depend on showing an altered pattern of haemoglobin attachment to the serum proteins. One technique employs paper electrophoresis of serum with subsequent differential staining of the strips to show the position of the protein bands relative to the haemoglobin pigments, and the other technique is based on a variation of the Schumm haemochromogen reaction (Schumm, 1912), in which the haemoglobin itself is involved.

The Schumm reaction, as normally performed, allows detection of methaemalbumin in much lower concentration than is possible by direct spectroscopic examination (Fairley, 1941). The presence of methaemalbumin in serum is indicative of intravascular haemolysis, of which the occasional occurrence in pernicious anaemia argues for a significant haemolytic component in that disease. The common finding of raised serum bilirubin levels in patients with the disease points to a similar conclusion, and it appeared possible that closer examination of such sera and specimens from other patients in whom haemolysis was occurring or suspected might yield information concerning the severity of the haemolytic process, and its response to therapy in relation to the patients' haematological and clinical progress.

Clinical Material

Cases studied included 10 cases of untreated pernicious anaemia, five cases of untreated megaloblastic anaemia associated with folic acid deficiency, nine cases of haemolytic anaemia of varying aetiology, three patients with evidence of minor blood transfusion reaction, and three cases of uraemia associated with anaemia in which haemolysis was suspected. In addition, tests were

performed on 18 patients with various pathological conditions, with or without anaemia, but in whom no haemolytic component was suspected. A control group of 30 sera from healthy individuals was also similarly investigated. As the underlying mechanism of the reaction became clearer an attempt was made to characterize further the implicated proteins. Tests were made involving urinary proteins from patients with nephrosis and a serum albumin fraction as supplied for transfusion purposes by the Lister Institute.

Experimental

Preparation of Haemoglobin Solution.—A stock solution of haemoglobin was obtained by lysing a known volume of washed normal red cells with an equal volume of distilled water followed by freezing and thawing to give an approximate concentration of 15 g. haemoglobin/100 ml. representing a dilution of 1 in 2. This solution may be preserved in the frozen state for several weeks.

Addition of Haemoglobin Solution to Test Serum.—Haemoglobin concentrations of approximately 0.3, 0.6, and 1.2 mg./ml. serum were obtained by the addition of 1 ml. test serum to 0.002, 0.004, and 0.008 ml. of the stock haemoglobin solution. This gives haemoglobin dilutions in serum equivalent to 1 in 1,000, 1 in 500, and 1 in 250 of the concentration normally present in packed red cells. A further 1 ml. serum was placed in a fourth tube to which no haemoglobin was added.

Although with practice these volumes were found to be adequate for spectroscopic examination in a Hartridge reversion spectroscope, double quantities are more convenient if the specimen permits.

Paper Electrophoresis of Test Serum.—Samples of each of the above sera, with and without added haemoglobin, after standing for 30 minutes at room temperature were subjected to electrophoretic separation on filter paper. Most separations were carried out on an apparatus of the "hanging-strip" type (Neale, 1955) using 0.05 ml. serum on Whatman No. 100 filter paper. The remainder were performed on an E.E.L. horizontal type apparatus using 0.02 ml. serum on Whatman No. 3MM paper. All separations were allowed to run for 16 to 18 hours at room temperature. Barbitone buffers of *p*H 8.6 were employed, as described by Durrum (1950) for the hanging type apparatus, and according to the formula in the manufacturer's handbook for the E.E.L. apparatus (30 g. sodium barbitone, 19.5 g. $CH_3COONa.3H_2O$, 205 ml. 0.1 N HCl, made up to 3 litres with distilled water). After electrophoresis the strips were dried at 100° C. and then cut longitudinally into two equal parts, one half stained for proteins with bromphenol blue and the other half retained for subsequent spraying with benzidine reagent to show the positions of haemoglobin or haem-containing pigments relative to the protein fractions.

Preparation of Haematin Solutions.—Haemin was prepared from human red cells according to the method described by Gattermann (1941). A solution of alkaline haematin was prepared freshly as required, by dissolving 10 mg. of this material in 10 ml. N/10 NaOH and diluting to 100 ml. with water as soon as solution was achieved. Portions of this dilute solution (0.1 mg./ml.) were added to 2 ml. portions of serum, giving final concentrations up to 10 μg. haematin/ml. serum. Electrophoresis of these mixtures was carried out as described above.

Benzidine Reagent.—This was prepared by adding 1 ml. of 20 volumes hydrogen peroxide to 30 ml. of a saturated solution of benzidine (extra pure for blood testing, B.D.H.) in glacial acetic acid. This ratio of peroxide to benzidine was found to give optimum sensitivity with minimal background discoloration. The mixed reagent was applied without delay to the half-strips by means of an all-glass spray, when an intense blue colour developed within a few seconds in the presence of haemoglobin or its haem-containing derivatives. This blue colour changes over the next few minutes to a blue-black, and the background, initially white, becomes light brownish-yellow. If a permanent record is desired the strip should be photographed as soon as possible after staining. In place of benzidine, which is now becoming difficult to obtain in a highly purified state because of its reported carcinogenic properties, *o*-tolidine may be used. The reagent prepared as described by Kohn and O'Kelly (1955), namely equal parts of a 4% ethanolic solution of *o*-tolidine, glacial acetic acid, and water, to which is added a $\frac{1}{4}$ volume of 20 volumes hydrogen peroxide, gives results similar to the benzidine spray, but the colour fades rather more quickly.

Schumm Reaction.—After samples have been removed for electrophoresis the four sera were covered with a layer of ether and 0.1 ml. AnalaR ammonium sulphide added to each as recommended by Fairley (1941). They were then examined in a Hartridge reversion spectroscope. In the presence of methaemalbumin a positive Schumm test is indicated by the appearance of a sharp band at 558 mμ; a weaker band at 530 mμ may also be seen. However, when haemoglobin is involved in the production of the haemochromogen the 558 mμ band only is seen.

Procedure for Urine.—Haemoglobin dilutions of 1 in 4,000, 1 in 2,000, 1 in 1,000, and 1 in 500 in urine were made and 0.2 ml. samples taken for electrophoresis; otherwise procedures were as for serum.

Results

Electrophoretic Behaviour of Haemoglobin and Haematin in Normal Sera.—In the absence of any slight haemolysis introduced during the taking of the blood or separation from the cells, normal serum is spectroscopically clear and no blue colour appears on spraying the electrophoretic strip with benzidine reagent. However, such slight haemo-

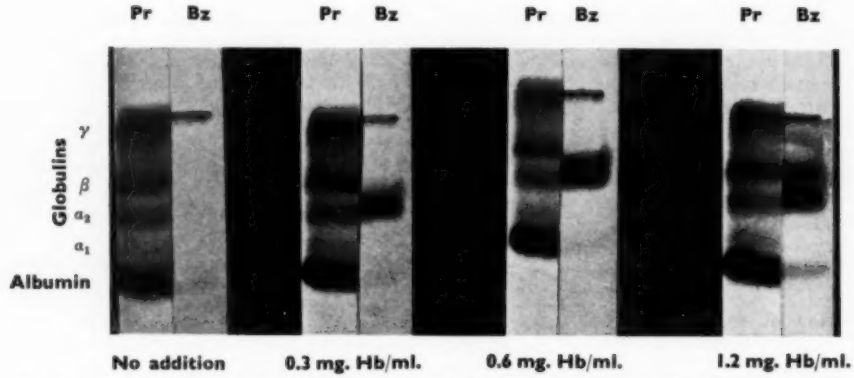

FIG. 1.—Electrophoretic results after the addition of haemoglobin to normal serum. Hb is carried by haptoglobin to a concentration of 0.6 mg./ml. Increasing this to 1.2 mg./ml. causes "overflow" into the β globulin and the albumin, and a trail is also left. Pr = protein stain. Bz = benzidine stain.

lysis is not always possible to avoid, even when the utmost care is taken, viz., permitting the blood to flow freely through a large needle (18 s.w.g.) without the use of a syringe, and allowing an adequate time for good clot retraction. When sufficient haemolysis occurs to permit the alpha band of oxyhaemoglobin to be just discernible spectroscopically in a 16 mm. test-tube, then the sprayed strip develops a diffuse blue band between the α_2 and β globulins. Occasionally this rather faint band is seen directly in line with the α_2 fraction. On deliberately adding the two lower haemoglobin concentrations defined above to most normal sera a progressive intensification of this diffuse band is seen. When the highest concentration, 1 in 250, is reached, however, this benzidine-staining band is seen to have two components, one in a position similar to the above, and the other, a slower moving band, corresponds to the faster moving β globulin fraction. At this concentration, too, a third though much less intense band may also be seen opposite the albumin and a diffuse trail of haemoglobin becomes apparent behind the β globulins extending back to the origin (Fig. 1). This composite pattern appears to reflect the over-saturation of the haemoglobin-carrying mechanism of normal serum, but in certain of the control sera, where the α_2 globulin appeared unusually high, separation into two bands did not occur but a further intensification of the single band was seen. On the other hand, in a few instances the appearance of a β globulin band began at a dilution of 1 in 500, although these were comparatively rare occasions.

Paper electrophoresis of haemoglobin solutions in saline at similar concentrations exhibited only a trail, quite dissimilar to the discrete bands obtained in the presence of serum. Coating the filter paper with protein by pre-running serum or an albumin transfusion preparation before applying the haemoglobin solutions did not materially alter this trail and did not produce the characteristic pattern seen when haemoglobin and serum are mixed before electrophoresis. The absence of this trail must indicate a firm combination with protein fractions which protects the haemoglobin from adsorption on to the paper (Fig. 2).

The binding of haematin to serum proteins was similarly studied, and it was found to be carried almost equally by the albumin and the leading β globulin fractions (Fig. 3). A similar result was reported by Fiser-Herman and Davorin (1953) with haematin prepared by alkali denaturation of haemoglobin, although they found haematin prepared from haemin was bound to α_2 globulin. Serum to which haematin had been added showed an absorption band at 624 mμ when examined in the spectroscope.

Schumm Reaction Applied to Normal Serum.—Schumm (1912) considered his test to identify haematin, but Fairley (1941) interpreted a positive Schumm reaction as indicative of methaemalbumin in the serum under examination. In non-haemolysed normal serum no absorption bands are seen following this reaction, whereas in sera to which small additions of haemoglobin have been made (dilutions of 1 in 1,000 and 1 in 500) the broad but rather faint band of reduced haemoglobin can be seen. This reverts to the double-banded oxyhaemoglobin spectrum on gently shaking with air, but the reduced spectrum returns on further standing. At the highest con-

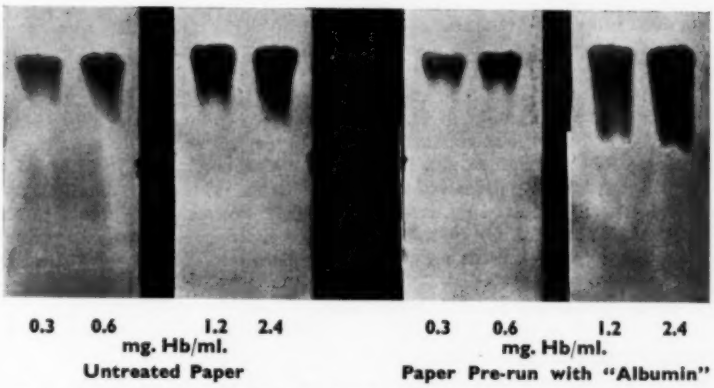

FIG. 2.—Electrophoresis of haemoglobin on albumin-coated and untreated paper. In the concentrations used Hb is clearly visible only after benzidine spraying and does not form a discrete band but is adsorbed on to filter paper whether or not this has been first " carpeted " with albumin.

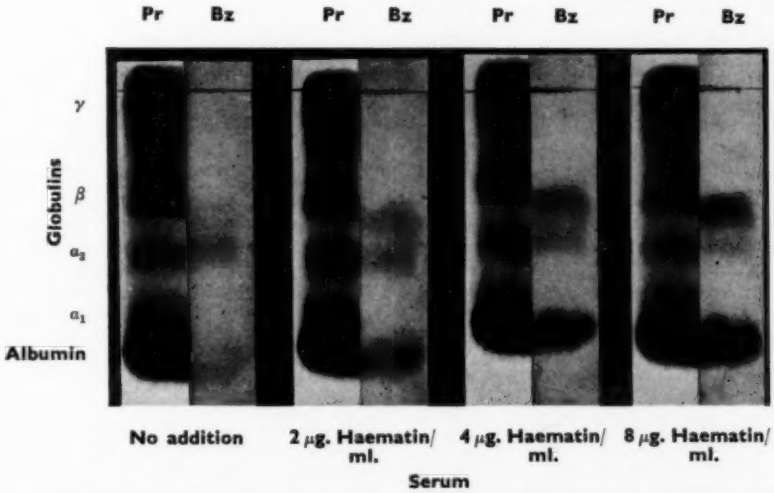

FIG. 3.—Electrophoresis of normal serum to which haematin has been added. Haematin is taken up by both albumin and β globulin. A small quantity of haemoglobin is present in this serum and migrates with the haptoglobin.

centration (1.2 mg./ml.) of haemoglobin this band may merely be intensified, but in some sera a new sharper and denser band at 558 mμ slowly appears through the centre of the reduced haemoglobin. More occasionally this band has been observed at the next lower concentration also, viz., 0.6 mg./ml. Reversion to the oxyhaemoglobin spectrum takes place on shaking in this case too, but ultimately a stage is reached when the 558 mμ band is permanent and irreversible.

Although this band appears at the same wavelength as in the classical Schumm reaction, it does not seem to represent the same haemochromogen because its reversible replacement by the oxyhaemoglobin spectrum shows clearly that its origin is not methaemalbumin, but that it derives indirectly from haemoglobin. The production of methaemalbumin by the incubation of haemoglobin with serum for two days was reported by Fairley and Bromfield (1937), but the haemochromogen now described appears within minutes and under entirely different conditions. The authentic Schumm reaction given by serum to which haematin has been added *in vitro* differs

also by appearing even more promptly and giving two absorption bands. These bands can also be caused to fade and reappear for a time by shaking, but no alternative bands take their place. The limiting concentration of haematin in serum that could be detected by paper electrophoresis, which required only 0.02–0.05 ml. serum, was 1 μg. per ml. By Schumm's test when viewed in a 16 mm. test-tube, which needs at least 1 ml. serum, it was 2 μg. per ml. Thus, not only has the electrophoretic technique about twice the ultimate sensitivity of spectroscopy but it will also give reliable results with a very much smaller specimen.

Electrophoretic Behaviour of Haemoglobin and Haematin in Sera from Patients with Haemolysis.—Haemolysis may present as an acute severe episode or may exist in a milder and chronic form upon which a more severe phase may be superimposed. Differing electrophoretic patterns reflect these varying degrees of lysis. In patients experiencing an acute haemolytic crisis a large quantity of methaemalbumin is usually identifiable directly by its absorption band at 624 mμ and these sera may also contain free haemoglobin in varying amount. The urine may contain haemoglobin and methaemoglobin if the threshold level is exceeded. Such urines on electrophoresis may be found to contain other serum protein fractions whose disappearance coincides with that of the haemoglobin pigments. Their presence indeed may be due to competition for reabsorption by the latter in the tubules (cf. Hardwicke and Squire, 1955) rather than the result of any transient kidney damage. In one such case of paroxysmal cold haemolysis this cycle could be provoked at will by immersing the patient's feet and legs in cold water for a short period. Urines of this type show a strongly positive benzidine-staining albumin, but, for reasons discussed later, this alone is not necessarily evidence for the presence of methaemalbumin although indeed it may well be present as was claimed by Hensley and Blackburn (1953). These sera from cases showing acute haemolysis after electrophoresis and benzidine spraying give an intense blue reaction in the albumin area and there may also be a much weaker band on the leading edge of the β globulins. Behind this latter band a trail of haemoglobin may be seen (Fig. 4). If haemoglobin is added *in vitro* to these sera, the only visible result is that this trail intensifies and spreads forwards as seen when haemoglobin in increasing concentrations is itself subjected to electrophoresis (see Fig. 2). In addition to the case of paroxysmal cold haemoglobinuria mentioned above, this type of acute phase was observed in one patient suffering from acquired haemolytic anaemia of the immunological type and in two patients with pernicious anaemia.

Examination of sera from patients in whom chronic haemolysis of lesser degree has been occurring reveals a modification of this pattern, in which the benzidine-positive material in the albumin band is diminished whereas that in the β globulin region is increased, and when haemo-

FIG. 4.—Position, after electrophoresis, of haemoglobin added to serum from a patient with acute haemolysis. In this case methaemalbumin is found, but neither haptoglobin or Hb-binding β globulin is demonstrable, added Hb combining with the albumin and also leaving a trail.

globin is added to these sera this difference is further emphasized. Simultaneously the staining intensity of albumin, β globulin, and the haemoglobin trail is increased (Fig. 5a). In some cases, where haemolysis introduced during the collection of the specimen is minimal, the pattern after electrophoresis is normal, and it is not until the first haemoglobin addition is made that an abnormal picture appears (Fig. 5b). Examples of this type have occurred in cases of familial acholuric jaundice, mild transfusion reaction, acquired haemolytic anaemias of various types, including those associated with the collagen disorders and the reticuloses, anaemias associated with the uraemic state, as well as in pernicious anaemia and megaloblastic anaemias associated

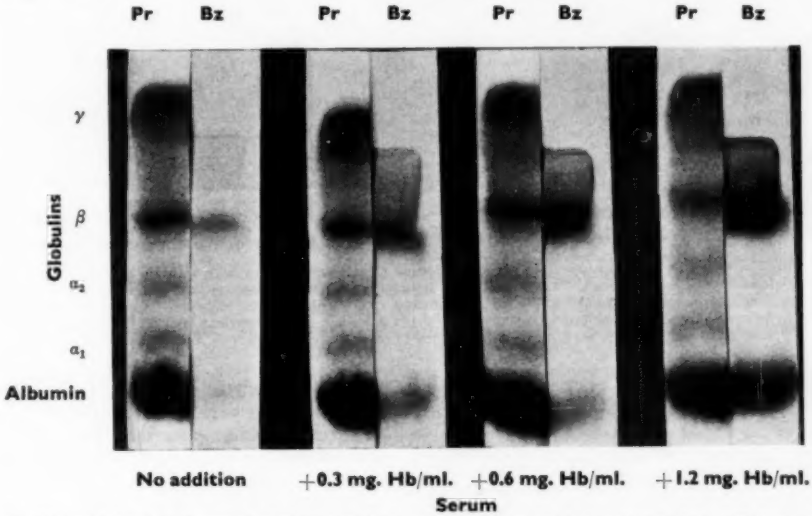

FIG. 5 (a).—Mode of attachment of added haemoglobin to sera from patients with chronic haemolysis. No haptoglobin is seen in this serum, haemoglobin being bound only to β globulin and albumin.

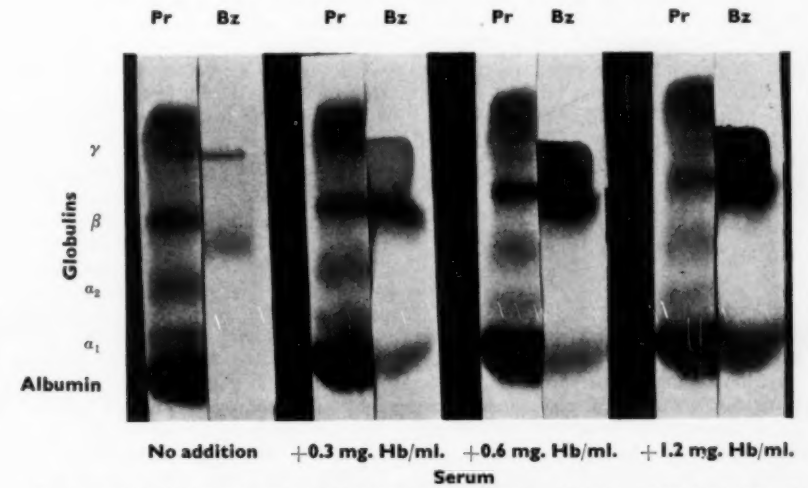

FIG. 5 (b).—A minimal quantity of haptoglobin is present in this serum: added haemoglobin is attached only to β globulin and albumin.

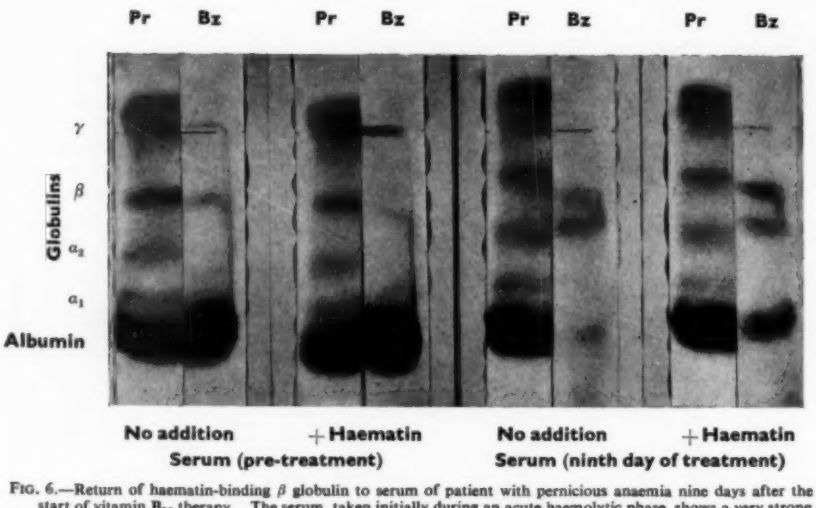

FIG. 6.—Return of haematin-binding β globulin to serum of patient with pernicious anaemia nine days after the start of vitamin B_{12} therapy. The serum, taken initially during an acute haemolytic phase, shows a very strong methaemalbumin band, but will not accept haematin into the β globulins. On the ninth day of treatment methaemalbumin has disappeared, a little haemoglobin is present, and is seen to be bound to both haptoglobin and β globulin. Added haematin is now accepted by β globulins as well as by albumin.

with folic acid deficiency. In many of these instances routine laboratory procedures had failed to demonstrate any evidence of an active haemolytic process. In addition to these pathological conditions, similar results were seen when cord blood was examined, reflecting the physiological haemolysis of the newborn.

The acceptance of haematin by sera of these groups also differs according to the severity of the attack. Its pattern of attachment to the albumin and β globulin fractions parallels their ability to bind haemoglobin; thus in the most severe cases, where both the α_2 and β globulin binding fraction had disappeared, the addition of haematin resulted in uptake by the albumin alone. This points to the probability of common binding sites in both albumin and β globulin for haematin and haemoglobin (Fig. 6). By following the progress of patients with pernicious anaemia under treatment with vitamin B_{12} the simultaneous return of the ability of the β globulin to bind both haematin and haemoglobin has been seen.

Schumm Reaction Applied to Sera from Patients with Haemolysis.—The character of the Schumm reaction in these sera was found to be dependent on the presence of methaemalbumin or quite small amounts of haemoglobin. If the former were present the haemochromogen spectrum described by Schumm (1912) and later by Fairley (1941) was at once obtained. However, in many cases where haemolysis was suspected this reaction was negative, and it was only after deliberately adding haemoglobin that a haemochromagen band appeared, although rather more slowly, a band at 558 mμ gradually replacing the reduced haemoglobin spectrum. This reaction has already been described as occurring in normal serum containing the higher haemoglobin concentrations, but in these abnormal sera the lowest concentration will invariably bring it about. At a haemoglobin dilution of 1 in 500 the band intensifies, but at 1 in 250, although still darker, its demarcation is a little less precise due to the broad absorption band of reduced haemoglobin with which its edges merge.

Influence of Therapy on Haemoglobin Distribution in Serum.—In order to follow the return to normal of the electrophoretic behaviour and Schumm reaction, a study of the influence of therapy with vitamin B_{12} in patients suffering from pernicious anaemia was chosen, as in this condition a good response to therapy could be confidently anticipated. For the same reason the progress of another group of patients with non-Addisonian megaloblastic anaemia responding to folic acid therapy was also followed. Both tests had always been found to be either normal or abnormal for any given serum, and further evidence that both were related to one common mechanism in haemoglobin carriage arose from

the simultaneous changes they underwent in response to therapy. This sequence of change may be illustrated by reference to the following case.

D. A., a woman aged 56, was admitted to hospital as a medical emergency. She had a slight icterus, severe anaemia, and was mentally confused. Apart from a systolic cardiac murmur, slight ankle oedema, and the spleen being just palpable, there was no other noteworthy clinical finding.

Blood examination showed severe macrocytic anaemia: Hb 36% (5.3 g.%), R.B.C.s 1.03 m. per c.mm., P.C.V. 12%, M.C.V. 116 cμ, reticulocytes 4.8%. Blood films showed features consistent with a diagnosis of pernicious anaemia. The bone marrow was megaloblastic and there was histamine-fast achlorhydria. The indirect van den Bergh reaction was positive, serum bilirubin was 2.4 mg./100 ml., Schumm's test was positive; fasting serum iron 182 μg./100 ml. and iron-binding capacity 230 μg./100 ml. Osmotic and mechanical fragility tests on the red cells were within normal limits and the direct Coombs test was negative.

Treatment with daily intramuscular injections of 100 μg. vitamin B_{12} was instituted, and within two days there was striking clinical improvement. Biochemically the first change noted was a sharp fall in serum iron to 20 μg./100 ml. 48 hours after treatment was started (Hawkins, 1955). On the fifth day of treatment the serum bilirubin had fallen to 0.5 mg./100 ml., reticulocytes had risen to 28.5%. On the seventh day the Schumm test was negative.

Studies of the electrophoretic pattern during the recovery phase revealed a gradual loss of benzidine-staining pigments in the albumin region and these had entirely disappeared by the seventh day, the same day as the Schumm test became negative. After haemoglobin addition the band in the β globulin region gradually became more intense, but there was no uptake of haemoglobin in the α_2 globulin area until the ninth day of treatment, and even then the ability of this fraction of the α_2 globulin to carry haemoglobin was far from normal, and remained so until the eleventh day of treatment (Fig. 7).

The absence of any haemoglobin binding in the α_2 and the minimal uptake in the β globulin region suggest that in this patient haemolysis had played a major role in the production of such a severe degree of anaemia. The initially high concentration of benzidine-staining compound in the albumin fraction was almost certainly due to the presence of methaemalbumin, although after haemoglobin additions some of this could have been accounted for by the haemoglobin. The simultaneous return of the Schumm test to normal and the disappearance of this albumin band in the serum after electrophoresis is very much in favour of this hypothesis. The fact that the test with ammonium sulphide after the addition of haemoglobin *in vitro* remained positive so long as there was any acceptance in the β region is strongly suggestive that it is the presence of this β-globulin-haemoglobin compound which is responsible for the formation of the haemochromogen.

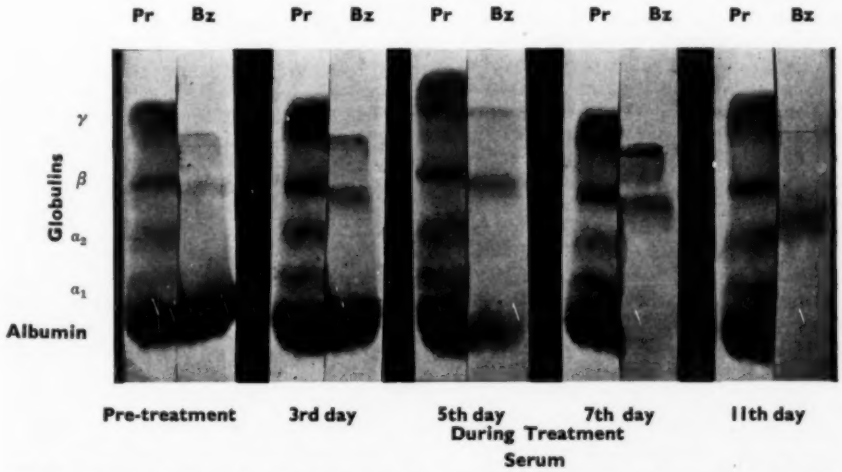

FIG. 7.—Characteristic changes found on electrophoresis of serum from a patient with pernicious anaemia undergoing treatment with vitamin B_{12}. Methaemalbumin disappears by the seventh day and haptoglobin reappears by the eleventh day.

A similar sequence of changes was noted in all the 10 cases of pernicious anaemia successfully treated with vitamin B_{12}, although with one exception they showed features of a more chronic rather than an acute haemolytic process.

Identical patterns were also seen in three patients in whom acute intravascular haemolysis had occurred, but who recovered either spontaneously or as a result of therapy, and four cases of acholuric jaundice subjected to splenectomy recovered their normal haemoglobin-protein attachments within seven to 10 days after the operation. In one patient suffering from a chronic haemolytic anaemia of unknown aetiology, splenectomy was undertaken after all other forms of therapy had failed to influence the anaemia. One week after this operation his serum, after adding to it haemoglobin *in vitro*, gave normal electrophoretic and chemical reactions, but after another week, however, he again showed the pre-operative biochemical abnormalities and a haematological relapse.

In all these patients a constant sequence of events was observed. The benzidine-staining material gradually disappeared from the albumin, and that in the β position increased until finally haemoglobin attachment to the α_2 fraction was detected. This then increased at the expense of the β-bound component until eventually only the sera containing the higher concentrations of haemoglobin, i.e., 1.2 and 0.6 mg./ml., showed any staining in the β region. The trail of free haemoglobin gradually diminished as these changes in the bound fractions proceeded. The speed of return to normal in treated cases of vitamin B_{12} and folic acid deficiency anaemias favours a central rather than a peripheral haemolytic mechanism in these conditions. It appears that haemolysis must cease very quickly on institution of therapy, a conclusion supported by the rapid fall in serum iron, certainly sooner than would be anticipated if lysis were to continue till the original peripheral cell population was replaced by normal cells. It seems more probable that the time required for cessation of haemolysis is of the same order as that taken for the return to a normoblastic marrow, and this does not seem to be coincidental.

Discussion

Amongst their multiple functions serum proteins have been shown to play a major role in the transportation of many substances normally found in human blood. Thus, iron was found to be carried by a β_1 globulin (Surgenor, Koechlin, and Strong, 1949; Holmberg and Laurell, 1947), transport copper is albumin bound (Bearn and Kunkel, 1954; Horst, 1954), vitamin B_{12} was observed to be combined with α and β globulin (Latner and Zaki, 1957), and during paper electrophoresis bilirubin migrates with albumin, to mention but some of the known combinations. It is usually assumed that these protein carriers merely hand on their load to the recipient tissues and are then free once more for further use; for example, the serum iron-binding capacity is the same before, during, and after an iron absorption test. However, the most likely explanation for the characteristic changes observed in cases of intravascular haemolysis is that liberation of haemoglobin into the plasma *in vivo* results in a reduction in the quantity of those plasma proteins with which it combines.

In 1939 Polonowski and Jayle demonstrated that if a small quantity of haemoglobin were introduced intravascularly it appeared to become attached to protein(s) in human plasma, the protein-haemoglobin compound then giving a positive peroxidase reaction. They called this protein haptoglobin and it was later found (Jayle and Abdellatif, 1946) to have a high carbohydrate content. Attention was drawn more recently to the error that it introduced into analysis of electrophoretic strips of haemolysed sera because of the retarding effect that haemoglobin has on α_2 globulin, resulting in an apparent increase in β globulin at the expense of the α_2 globulin (Wieme, 1953, 1954; Tuttle, 1955). The former author described how with increasing deliberate additions of haemoglobin first one then two bands appeared, but these were visible to the eye and were produced by far greater amounts (additions of 4 to 40 mg./ml. serum) than have been used here. These two bands probably represented α_2 globulin uptake (the haptoglobin of Jayle) and free-moving haemoglobin, but the amount added was too great to have been of value in discriminating between sera from normal individuals and those undergoing haemolysis.

If the primary haemoglobin carrier is the haptoglobin fraction in the α_2 globulins, as suggested by Jayle and Boussier (1955), any condition which caused an increase in the α_2 globulins might also increase the ability of the serum to bind haemoglobin. Their investigations of sera from patients suffering from acute and chronic infections, malignancy, and disorders in which severe tissue damage occurs and which characteristically exhibit a rise in α_2 globulin, showed that these sera also accept larger quantities of haemoglobin in that fraction than do sera from normal persons.

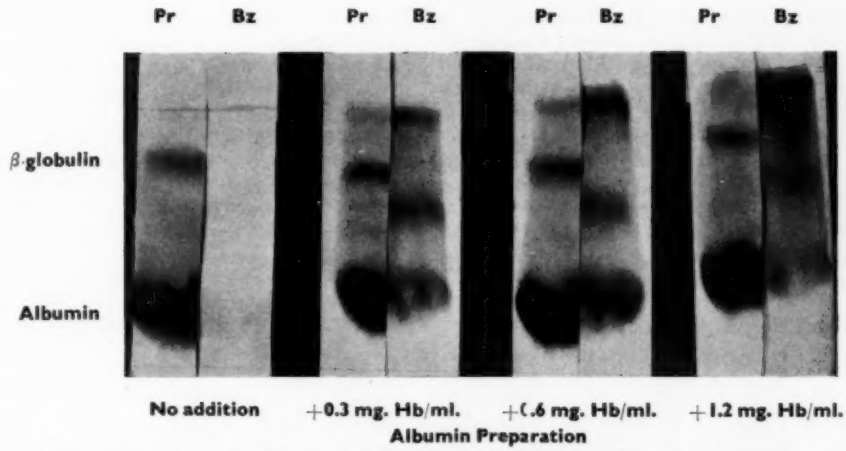

FIG. 8.—Addition of haemoglobin to albumin transfusion preparation, showing presence of an α_2-globulin binding fraction. Haemoglobin is attached also to albumin, but does not combine with the β globulin in this preparation.

From our own results with normal sera it appears that in different individuals there is sufficient haptoglobin to combine with haemoglobin concentration ranging from about 0.3 to 1.2 mg./ml. In some control sera where the α_2 globulin concentration was unusually high, 2.4 mg./ml. haemoglobin was found insufficient to form more than one electrophoretic band. The high α_2 globulin concentration which occurs in the nephrotic syndrome did not saturate till even more haemoglobin had been added. Conversely when the concentration of α_2 globulin is decreased, acceptance of smaller than normal quantities of haemoglobin should be demonstrable, and in the control group one individual was found whose haptoglobin was barely able to carry a concentration of 0.3 mg./ml.

Once the haptoglobin has been saturated, electrophoresis shows another slightly slower-moving band caused by secondary acceptance by a β globulin fraction. This can be shown to be a β_1 globulin by performing the electrophoresis in a buffer containing 20 mg./100 ml. calcium ion, which was shown by Laurell, Laurell, and Skoog (1956) to retard the β lipoprotein and resolve the β globulins into two bands. The usual E.E.L. buffer was employed to which calcium chloride had been added to a concentration of M/200. In an attempt to characterize this fraction further, experiments were carried out with a sample of pure iron-free transferrin,* but no attachment of haematin to this protein was seen. Likewise an albumin transfusion fraction containing β globulin which, by its ability to transport ^{59}Fe was shown to include transferrin, did not contain any β-haemoglobin-combining protein (Fig. 8). There was, however, evidence of haemoglobin attachment to an ill-defined protein of approximately α_2 globulin mobility.

Haemoglobin forms a complex with β_1 globulin only after saturating the haptoglobin, but in haemolytic sera this form of attachment is seen with even the minimal addition of 0.3 mg./ml. and this must, therefore, represent a depletion of haptoglobin. In one or two instances of very severe haemolytic activity it has been possible to show that both the α_2 and β globulins are incapable of binding haemoglobin, and this is further evidence of the hypothesis that in disposing of extracorpuscular haemoglobin the protein attached to it is compulsorily sacrificed, the β-binding component also having been lost. A similar conclusion is reached with regard to haptoglobin by Nyman (1957) and Laurell and Nyman (1957), who recently found, using a paper electrophoresis technique similar to our own, that it is absent from sera in cases of pernicious anaemia and returns on treatment with vitamin B_{12}. Their conditions of electrophoresis, however, may have caused an overlap of free-moving haemoglobin and the β globulins, which could explain how the haemoglobin complex with this fraction has escaped notice. The same may well hold for the report by Liang (1957) that human serum containing haemoglobin exhibits but three bands after electrophoresis, namely, albumin-haemoglobin, α_2 haemoglobin, and free-moving haemoglobin.

* Obtained through the kindness of Professor Schultze, of the Behringwerke, Marburg, Germany.

Although no quantitation was attempted in this series, there appeared to be reduced amounts of α_2 and β globulins even on qualitative inspection of the strips, but it was impossible to make any similar visual assessment of the albumin. It is significant that Christenson and Dacie (1957) have reported a diminution in these fractions in sera of patients who formed warm auto-antibodies.

In the following way it was possible to demonstrate quite clearly that the effects being seen in these sera were due to a serum protein deficiency and not to any peculiarity of the patient's haemoglobin. A constant addition of haemoglobin was maintained throughout a series which consisted of: (1) normal serum and normal haemoglobin, (2) normal serum and patient's haemoglobin, (3) patient's serum and normal haemoglobin, and (4) patient's serum and patient's haemoglobin.

The normal and patient's sera were also examined without added haemoglobin. Fig. 9

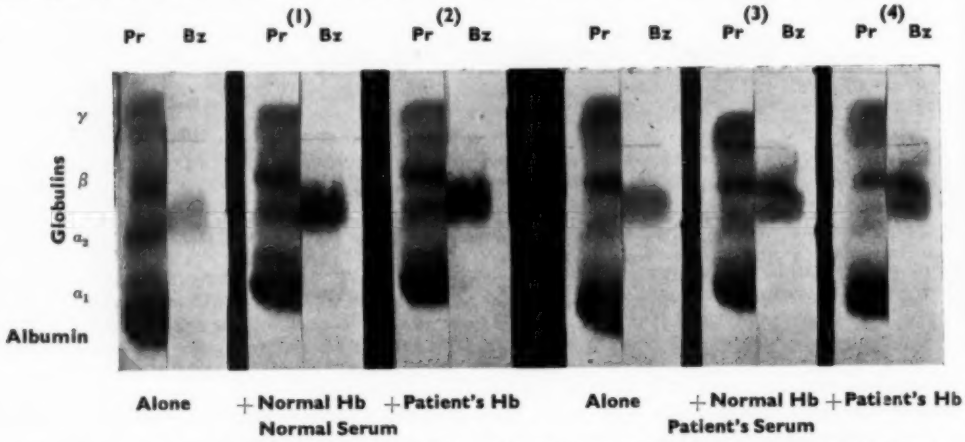

FIG. 9 (a).—Result of electrophoresis of mixtures of haemoglobin and sera from normal individuals and subjects showing haemolysis. Normal serum shows haptoglobin-haemoglobin complex with either haemoglobin; patient's serum exhibits a small amount of haptoglobin-haemoglobin complex and β globulin binding.

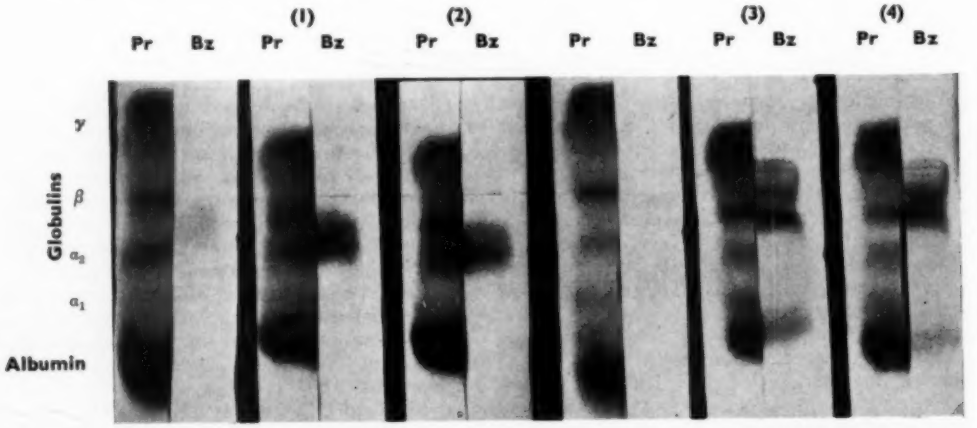

FIG. 9 (b).—Normal serum shows haptoglobin-haemoglobin complex, patient's serum is devoid of haptoglobin, but β globulin and albumin binding is seen, also a haemoglobin trail.

shows the result of electrophoresis. Both (1) and (2) gave a normal result and negative haemochromogen reaction, whereas (3) and (4) gave an abnormal result and positive haemochromogen reaction. Fig. 9 also illustrates typical quantitative differences that are found in sera from individuals showing evidence of haemolysis. In Fig. 9a (3) and (4) is shown haemoglobin attached to a small amount of haptoglobin, to β globulin, and a slight trail unattached to protein. Fig. 9b (3) and (4) shows complete absence of haptoglobin binding, but strong β globulin and slight albumin uptake. The free-moving haemoglobin is clearly visible behind the β-globulin-haemoglobin complex. Other sera of this type were tested and similar results obtained. It was also found that if to a serum which gave such abnormal results an equal volume of normal serum was added before the haemoglobin addition, then the mixture reacted as normal serum. These experiments show clearly that a factor, initially lacking, was supplied to the abnormal by the normal serum, and that there must have been in the latter a sufficient quantity of haptoglobin to prevent the mixture giving an abnormal reaction.

Reference has already been made to the appearance of a "haemolytic" pattern in a number of cord bloods taken from normal full-term infants. Tuttle (1955) showed that the sera from newly born babies demonstrated a different haemoglobin-carrying mechanism from that observed in the adult. He found very little haemoglobin attached to the α_2 fraction but a considerable amount was taken up in the β globulin, and that this pattern gradually changed in the first week of life to that normally found in older subjects. It is almost certain that what he was studying was the electrophoretic pattern of increased haemolysis which occurs normally in the early days of life, reflecting the shorter life span of red cells containing foetal haemoglobin. A small number of cord bloods from "Rhesus babies" were also examined and, not surprisingly, showed marked haemolytic patterns.

The Schumm reaction as conventionally employed depends on the production of a haemochromogen from an albumin-haematin complex under the influence of ammonium sulphide. Haematin is also accepted by β globulin, but since apparently equally strong, though not preferential, albumin binding always occurs simultaneously in serum, it is not possible on the present evidence to say what part this β globulin takes in the classical Schumm reaction. The haemochromogen which is described here appears to derive from the β_1 globulin-haemoglobin complex under the same chemical treatment. This becomes apparent on carrying out both electrophoretic and chemical tests concurrently on a large number of sera. This haemochromogen is produced only when there is sufficient haemoglobin to "spill over" into the β globulins. As long as it is all covered by the prime binding protein, haptoglobin, a spectrum of reduced haemoglobin only is seen on ammonium sulphide treatment. Although binding to albumin occurs to a small degree as the haemoglobin concentration is increased, this albumin-haemoglobin complex has probably no part in the haemochromogen reaction, for while formation of an albumin complex may be induced in protein-containing urine, no haemochromogen is produced unless there is also a β globulin linking protein, and this was found in only two of four nephrotic urines examined (Fig. 10). Jayle and Boussier (1954) found that among 31 nephrotic patients examined only one child excreted a significant amount of haptoglobin. If large quantities of the haptoglobin were lost in this way, false positive tests for haemolysis might well complicate the serum studies on these patients. It appears likely that many Schumm reactions that have been reported positive in the past have in fact been contributed to by the haemochromogen reaction now described, as most plasmas in acute haemolytic crises contain *in vivo* more than sufficient free haemoglobin to provoke it. Also a naturally occurring mixture of haemoglobin and sulphaemoglobin reacted both electrophoretically and with ammonium sulphide exactly as haemoglobin itself.

The fate of these various haemoglobin-protein complexes in the body is not clear, but it appears reasonable to assume that they are removed complete from the blood stream by the reticulo-endothelial system, the haemoglobin broken down, and the associated serum protein lost from the circulation.

The two tests described in this paper are always found to be complementary, never contradictory. They are positive only if there is insufficient haptoglobin to bind all the haemoglobin present. In practice it was found that a concentration of 0.3 mg. haemoglobin per ml. serum was critical in differentiating normal serum from that of patients experiencing haemolysis. For routine work when there is an adequate sample, the test with ammonium sulphide is quicker to carry out than the electrophoretic technique. When the sample is very small it is then often possible to obtain an answer by electrophoresis without further haemoglobin additions, since it commonly happens that circum-

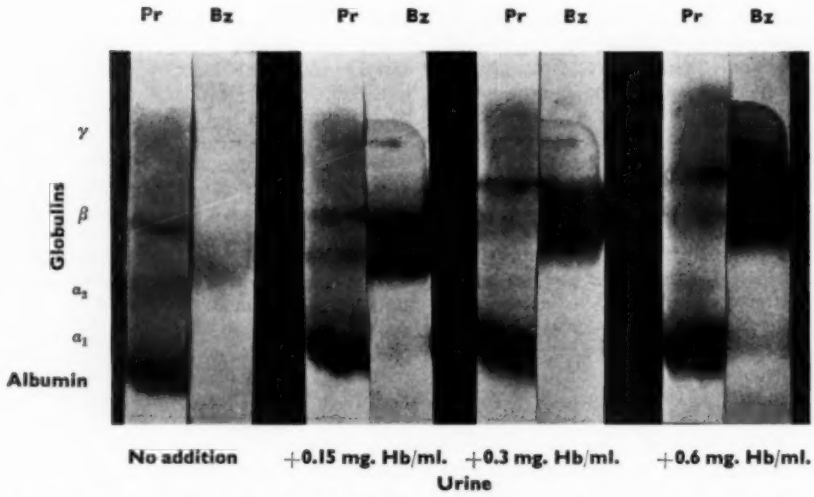

FIG. 10.—Addition of haemoglobin to urine of two patients with the nephrotic syndrome; (a) electrophoresis shows marked haptoglobin, but minimal albumin binding of haemoglobin.

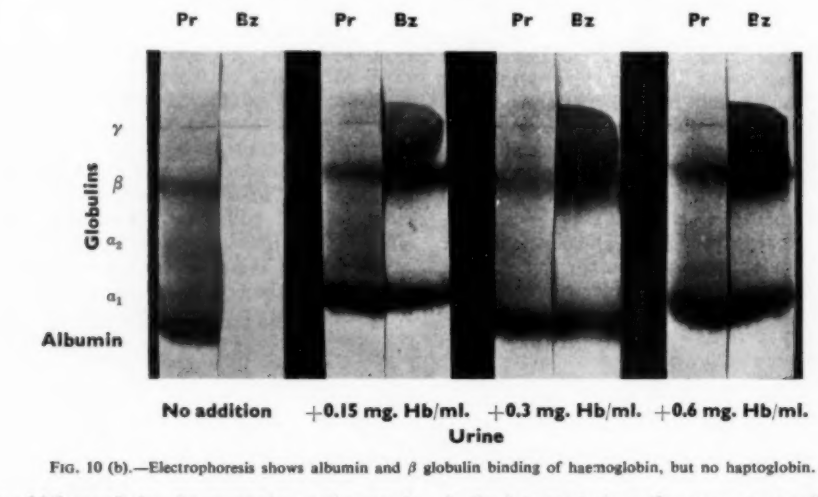

FIG. 10 (b).—Electrophoresis shows albumin and β globulin binding of haemoglobin, but no haptoglobin.

stances which preclude a large specimen also result in the introduction of haemoglobin incidental to collection of the specimen. Familiarity with the electrophoretic pattern and experience of depth of staining will often enable one to form an opinion of the probability of haemolysis even under these unfavourable circumstances. For example, if no α_2 but only β-globulin binding can be detected, then it is fairly safe to presume that haemolysis has occurred even if the precise amount of added haemoglobin is unknown. Easier still, of course, is the interpretation of a very strong albumin and β-globulin stain which is most likely to be due to haematin complexes although it may be partly caused by haemoglobin, in which case a trail of this pigment must be visible too. Finally, it seems pertinent to consider that comparable clearance mechanisms may also exist for other plasma constituents and that the haemoglobin disposal system may be but one example of this type. This form of serum transportation, in which the protein is compulsorily lost with its load, appears a novel concept,

and perhaps applies only where immediate sequestration of a "foreign" protein is essential, and there may be a close analogy between this system and those of the antigen-antibody type. Further examples may be found once the principle is conceded, and these will help to decide whether the haemoglobin interactions are unique or can be better classed as a "transport" or as a "defence" system.

Summary

Two complementary tests are described which detect intravascular haemolysis and are diagnostic when other simple tests are equivocal or even negative.

These two tests, one electrophoretic, the other purely chemical, are based on a common principle, the mode of haemoglobin attachment to serum protein. Electrophoretic evidence is presented that haemoglobin can form complexes not only with haptoglobin but also with a β globulin and albumin.

Application of the tests to a variety of clinical conditions involving haemolysis has shown that disappearance of haemoglobin from plasma is accompanied by loss of its carrier proteins. The implications of this fact are discussed.

Serial investigation of patients with megaloblastic anaemia treated with vitamin B_{12} or folic acid show that haemolysis ceases very soon after institution of therapy and points to a central rather than a peripheral haemolytic mechanism.

The authors wish to express their gratitude to Dr. J. N. Marshall Chalmers for his interest and encouragement throughout this work and for his criticism of the paper, to Professor J. R. Squire for reading the paper, and to Dr. H. G. Kohler for specimens of blood from patients with anaemias of pregnancy.

Addendum

Since submitting this paper for publication, Allison and ap Rees (1957), using the starch-block electrophoretic technique previously employed by Smithies (1955), have described experiments with serum from normal individuals and from patients in whom haemolysis had occurred. Their results show an acceptance of up to 135 mg. haemoglobin/100 ml. serum in the normal case, but a marked reduction or complete absence of haptoglobin in the serum of the patients studied. They concluded, in accord with Laurell and Nyman (1957), that both the serum haptoglobin level and tubular reabsorption of haemoglobin together determine the renal threshold for haemoglobin.

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THE VALUE OF LABORATORY TESTS IN HYPOTHERMIA

BY

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The biochemical changes which have been reported during and after hypothermia for cardiac surgery, and on which the literature is now considerable, are similar in man to those already found in animals. However, there are comparatively few references in the literature to investigations carried out in neurosurgical units, those of Gunton, Scott, Lougheed, and Botterell (1956) and Gray (1957) being the more comprehensive. The aim of the present investigation has therefore been to decide whether there are changes in the biochemistry in cranial as compared with cardiac surgery, what screening tests, if any, should be carried out in patients in whom hypothermia is indicated, and if any useful purpose is served by following up those cases with a view to anticipating any of the recognized or theoretical complications, to which reference will be made later.

Method

Investigations were carried out on 38 patients who were submitted to craniotomy, 34 for direct attack on intracranial vascular abnormalities and four for removal of a meningioma. The studies originally did not set out to be exhaustive and have thus not all been performed on the same number of cases. These have been compared with a control series of 10 patients who had undergone craniotomy without hypothermia, but in none of whom was there a vascular abnormality as all such cases were operated on under hypothermia. Venous blood samples were collected at normal body temperature before the operation, during hypothermia at about 27.5° C. (81.5° F.), and on the day after operation when normothermic conditions had once more been established. Twenty-four-hour urine collections were also made for cation estimations pre- and post-operatively. Serum sodium and potassium were estimated using a flame photometer (Domingo and Klyne, 1949), bicarbonate (Van Slyke and Cullen, 1917), calcium (Tisdall, 1923), urea, using Nessler's reagent, sugar (Folin and Wu, 1920), amylase (Somogyi, 1941), total protein by the specific gravity method, and fibrinogen (Hiller, McIntosh, and Van Slyke, 1927). Quick's one-stage method was used to estimate the prothrombin time, the clotting time was estimated by Dale and Laidlaw's (1911) shot method, and the platelet count was done by Lempert's modification of Kristenson's technique (Darmady and Davenport, 1954).

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Results

Table I shows the average values found during the biochemical investigations in patients undergoing craniotomy with and without hypothermia. It will be seen that there was no alteration in the serum sodium level, though a few values outside normal limits were encountered. The changes in potassium were relatively small, and, although only once during and in four cases after hypothermia did the level drop below 3.9 mEq./l., in comparison with cases where hypothermia was not used a significant difference was noted. The bicarbonate picture presented the greatest variations, there being a significant decrease, both during and after hypothermia, which was absent when this form of anaesthesia was not used. The concentration of calcium increased during hypothermia, returning to the pre-operative level subsequently. There was little difference in the 24-hour urinary excretion of sodium and potassium with or without hypothermia. A few serum magnesium studies were also carried out, but are as yet inconclusive and therefore being further pursued.

Significant changes were also noted in the levels of blood sugar and urea in patients under hypothermia. There was a consistent and marked increase in the sugar concentration during hypothermia with a marked post-hypothermic drop, though with an incomplete return to the pre-operative level. The blood urea showed a steady rise during and after hypothermia, with values of up to 65 mg. per 100 ml. being recorded. The use of hypothermia also led to a small reduction in the total concentration of protein during and after operation with corresponding changes in the electrophoretic pattern.

TABLE I
AVERAGE BIOCHEMICAL FINDINGS IN HYPOTHERMIA CASES

	Na (mEq./l.)	K (mEq./l.)	HCO_3 (mEq./l.)	Ca (mg. per 100 ml.)	Urea (mg. per 100 ml.)	Sugar (mg. per 100 ml.)	Amylase (units)	Protein (g. per 100 ml.)	Urinary K (g. per 100 ml.)	Urinary Na (g. per 100 ml.)
Twenty-four hours before	140	5·0	28·0	9·2	25	103	122	7·3	2·23	3·33
Taken at 27·5° C. ..	138	4·6	24·7	10·1	30	197	122	7·0	—	—
Twenty-four hours after ..	139	4·5	24·9	9·3	35	144	126	7·0	2·20	1·99
Neurosurgical control series										
Before operation ..	138	4·8	27·5	9·4	22	97	114	6·7	2·60	3·38
During ,, ..	139	4·8	27·0	9·2	24	126	113	6·8	—	—
After ,, ..	142	4·9	27·5	9·0	24	103	116	6·7	1·88	2·11

Table II shows the results in the investigation of the haematological picture. No significant changes could be demonstrated as resulting from the use of hypothermia.

TABLE II
AVERAGE HAEMATOLOGICAL FINDINGS IN HYPOTHERMIA CASES

	Prothrombin Concentration (%)	Coagulation Time (min.)	Serum Fibrinogen (g. per 100 ml.)	Platelet Count (Thousands)
Twenty-four hours before	97	3	0·31	235
Taken at 27·5° C. ..	95	2	0·32	227
Twenty-four hours after	91	3	0·32	227
Neurosurgical control series				
Before operation	95	2½	0·33	220
During ,,	98	2	0·30	238
After ,,	80	3	0·34	240

Discussion

It will be seen that with regard to the serum sodium and calcium levels, our findings are in accord with those of most workers, though Churchill-Davidson (1955) has observed a rise in plasma calcium levels only below 25° C. (77° F.). It is with the potassium and bicarbonate concentrations that one notes conflicting views. Brewin, Nashat, and Neil (1956) have noted during thoracotomy a metabolic acidosis due to an increase in lactic acid and accompanied by a corresponding reduction in bicarbonate. Deterling, Nelson, Bhonslay, and Howland (1955) and Stone, Donnelly, and Frobese (1956), on the other hand, have demonstrated a respiratory acidosis due to a reduction in respiratory activity at decreasing temperatures leading to CO_2 retention and a fall in blood *p*H. It has also been shown that hypothermia is accompanied by hypokalaemia (Swan, Zeavin, Holmes, and Montgomery, 1953; Jaulmes, Delga, Bénitte, and Richard, 1955; Boeré, 1956; Pratt and Collins, 1956) presumably due to migration of potassium from the serum into the cells. On rewarming the reverse takes place, as well as an increase in potassium excretion in the urine. As, however, any operation results in an increased potassium and decreased sodium urinary loss owing to adrenocortical stimulation (Graham, 1954), the changes noted after hypothermia by other workers are therefore probably not specific. These studies have failed to show any increase in potassium excretion due to hypothermia.

It is in the light of the possible complications and danger of hypothermia that the results of the investigation have to be considered.

Increased Myocardial Irritability.—Swann *et al.* (1953) reported that respiratory acidosis associated with reduced serum potassium increases the incidence of ventricular fibrillation in dogs. Another observation was that the hypothermic myocardium in ventricular fibrillation loses potassium and that injections of potassium, prostigmine, or acetylcholine into the coronary circulation, as well as vagal stimulation, have a marked antifibrillatory effect. Several workers (Scurr and Organe, 1956; Swan, 1956; Gray, 1957) therefore advocate the use of hyperventilation with the production of a respiratory alkalosis in an endeavour to reduce the incidence of fibrillation and perfusion of the coronary circulation with potassium or prostigmine to defibrillate the resistant cold heart. Although in the present series a slight reduction in serum potassium accompanied a mild acidosis, there was not a single instance of ventricular fibrillation. This confirms the findings of the Liverpool Neurosurgical Unit, where in 180 consecutive craniotomies under hypothermia no cases of fibrillation were recorded (Gray, 1957), and in Toronto (Gunton *et al.*, 1956) where, in only two cases of fibrillation noted, hypotensive agents were used concurrently. Therefore, provided that the pre-operative levels of potassium and bicarbonate are normal, the dangers of in-

creased myocardial irritability in neurosurgical practice are negligible, even in the absence of hyperventilation, which was not employed.

Hyperelectrolytaemia.—Allott (1957) described several cases of bulbar poliomyelitis showing greatly increased serum sodium and chloride levels, with a greatly reduced urinary output of these ions and retention of urea. A similar biochemical picture has also been described in a number of intracranial lesions, notably tumours and ruptured berry aneurysms, both conditions in the surgical treatment of which hypothermia is increasingly being employed. Recently a patient was seen suffering from a subarachnoid haemorrhage who died from irreversible electrolyte changes, similar to those described by Allott, before an operation could be carried out. Although this rare complication, whose cause is uncertain, has not yet been described in hypothermia, the possibility should not be overlooked, particularly when dehydration and cessation of urinary excretion are present demanding prompt and intensive treatment.

Disturbed Liver Function.—As hypothermia reduces cell metabolism of the whole body, so liver function also is progressively reduced with a lowering of body temperature, glycogenesis, for instance, virtually ceasing below 31° C. (88° F.). Fisher, Fedor, Lee, Weitzel, Selker, and Russ (1956) have shown in experiments on dogs that after six hours of hypothermia there is a significant reduction in the glycogen and increase in non-protein-nitrogen content of the liver with some delay before full recovery on re-warming. Other workers have noted increases in the levels of blood sugar (Wynn, 1954) and urea (Talbott, 1941; Ciocatto, Brena, Fava, and Marocco, 1953) during hypothermia, which is in line with our findings. In two of our patients, serial blood sugar estimations carried out at two-hourly intervals during rewarming showed a progressive reduction in the sugar concentration with increasing temperature. This hyperglycaemia, which is constant and unaccompanied by ketosis or glycosuria, is therefore reversible and apparently without effect on the hypothermic patient, though this may not be true of someone who is diabetic, where hypothermia may lead to ketosis with consequently greater danger of myocardial irritability.

There are very few reports in the literature of protein studies during hypothermia and apparently none where electrophoresis was also carried out. Although this series demonstrated no significant changes in the total protein concentration, the relatively few electrophoreses so far carried out showed changes in the globulin during hypothermia which were not present when hypothermia was not employed. Further studies are now being carried out.

Brewin, Gould, Nashat, and Neil (1955) have shown in dogs that complete circulatory arrest for 10 minutes at 26° C. (79° F.) produces a profound disturbance of galactose removal, with correspondingly severe histological changes due to venous congestion and anoxia. Similar but less severe changes were seen in patients undergoing thoracotomy. This resulted in a progressive increase in lactacidosis during rewarming and jeopardized recovery. It was therefore felt that prevention of visceral congestion would prevent this metabolic upset. This infrequent complication cannot occur in patients undergoing craniotomy, as only local circulatory occlusion is practised. Histological studies in patients in this series who died have demonstrated no abnormalities in the liver.

Acute Pancreatitis.—Smith (1942) described several cases of acute pancreatitis developing in patients following the use of hypothermia. Histological studies revealed an incidence of minor degenerative changes of the pancreas in nearly 10% of his cases. No definite cause could be put forward, but it was thought to be due to autodigestion, the decrease in enzymatic activity of the pancreas not keeping pace with tissue resistance through relative anoxia from diminished blood flow. Although this complication has not been described elsewhere, pancreatic activity was investigated by estimating the serum amylase. It will be seen from the results that no significant changes were demonstrated; in only one patient did the diastase level reach 170 Somogyi units post-operatively. It may therefore be concluded that pancreatitis is unlikely to become a complication of hypothermia.

Decreased Blood Coagulability. — Various authors, notably Talbott (1941), Cahn (1953), Deterling *et al.* (1955), and Gray (1957), have reported an increased tendency to bleeding during and following hypothermia, due to an upset of the clotting mechanism. A number of cases of intractable post-operative wound oozing, some fatal, have been described, but as in several of these associated lesions, such as cirrhosis of the liver, were also present, a good cause for incriminating hypothermia has not been made out. Nevertheless, as a reduction in the platelet count and prothrombin concentration and an increase in coagulation time have been mentioned as

occurring following hypothermia, a series of investigations on these was carried out. As will be seen from Table II, the changes that did occur were negligible and not significant. Clinically no abnormal bleeding was noted either during or after hypothermia. Nevertheless, until there is absolute proof to the contrary, any condition leading to impairment of the clotting mechanism, such as liver damage, should be considered a contraindication to hypothermia.

Conclusions

These results have shown significant differences in the electrolyte picture. Neurosurgical cases are much less prone to acid-base imbalance, which stimulates ventricular arrhythmia, less likely to develop liver dysfunction and in consequence also less prone to show abnormal bleeding tendencies. Nevertheless, the following tests are worth carrying out: the serum potassium and bicarbonate, before and during hypothermia, as a guide to correcting an acidosis, though the most sensitive measure of myocardial irritability is still the E.C.G., and blood sugar to exclude diabetes. All other biochemical investigations and tests of clotting function of blood are only indicated when complications unassociated with hypothermia are suspected. It will therefore be seen that, as far as can be judged at present, the only danger of hypothermia that can be anticipated by invoking the aid of the laboratory is ventricular arrhythmia, but one can thus also exclude from hypothermia those patients in whom this procedure is contraindicated.

Summary

Investigations were carried out on 38 patients undergoing craniotomy under hypothermia for the treatment of vascular abnormalities and benign tumours, to demonstrate possible differences in biochemical and haematological findings compared with cardiac surgery and evaluate any screening tests that might be employed to anticipate any of the recognized or theoretical complications of hypothermia.

Significant changes were found in the serum potassium, bicarbonate and protein and blood sugar and urea concentrations, but not in serum sodium, calcium, magnesium, fibrinogen and prothrombin concentrations, coagulation time, and platelet count. These findings were compared with those described in cardiac surgery and it was concluded that neurosurgical cases are much less prone to acid-base imbalance, stimulating ventricular fibrillation, less likely to develop liver damage and in consequence less prone to abnormal bleeding tendencies.

The tests that are worth carrying out are the serum potassium and bicarbonate determinations before hypothermia as a guide to correcting an acidosis and the blood sugar to exclude hyperglycaemia. Other tests are only indicated when complications unassociated with hypothermia are suspected. The only danger of hypothermia that can be anticipated by carrying out the above tests is ventricular arrhythmia, though one can exclude from hypothermia those patients in whom this procedure is contraindicated.

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THE ROUTINE ASSAY OF B_{12} IN SERUM

BY

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The occasional request for the estimation of vitamin B_{12} in serum presents a difficult problem to the routine laboratory. In designing a suitable method to give reliable results when used at infrequent intervals the following modification of a technique described by Spray (1955) has been devised. It was irregularities in the response of the test organism, *Lactobacillus leishmanii*, as determined by replicate assays, which led to the alterations described. These irregularities were traced to slight differences in inoculum size, to errors in the measurement of small volumes of the standard B_{12} solutions, and to unequal agitation of the culture tubes when incubated in a mechanically stirred water-bath. This last irregularity led to the observation that *Lactobacillus leishmanii* grows better in unaerated or anaerobic conditions. The modified technique described below has, in our hands, given reproducible results in assays carried out at very long intervals, even as long as five months.

Method

Glassware and Metal Caps.—At the outset, new glassware was purchased, and, after cleaning in 10% hot nitric acid followed by thorough rinsing in distilled water, it was allotted to and kept for specific uses. All cleaning equipment, such as test-tube brushes, are likewise kept for one purpose only. Between each assay the same rigid procedure is adhered to. In this way contamination with vitamin B_{12} is avoided or, at least, kept to a minimum.

The aluminium caps for the test-tubes were first washed by boiling in water to which a detergent had been added and then well rinsed in distilled water. Between each assay they are simply boiled and rinsed in distilled water. Care must be taken in the cleansing of these caps, for during autoclaving some of the medium is deposited on the cap, a further source of error.

Organism.—The organism used is *Lactobacillus leishmanii* (N.C.I.B. 7884).

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Medium.—The medium is similar to that recommended by Spray (1955), and for convenience is made up in the following manner.

Solution A

Acid-hydrolysed casein, sufficient to give	2.7 g. total nitrogen
Sodium acetate, hydrated (anhydrous)	10 g. (6 g.)
KH_2PO_4	2.5 g.
K_2HPO_4	2.5 g.
Tryptophane	100 mg.
Cysteine HCl	100 mg.
Adenine	10 mg.
Guanine	10 mg.
Uracil	10 mg.
Xanthine	10 mg.
Salt solution B	20 ml.
Distilled water to	400 ml.

The acid-hydrolysed casein and salts are mixed with part of the water, heated to 70° C., and filtered. The tryptophane, cysteine HCl, and adenine are dissolved in a little N HCl, and the xanthine, guanine, and uracil in a little N NaOH and then added to the filtrate. This solution is stored under toluene in the cold. It keeps for at least three months.

Salt Solution B

$MgSO_4.7H_2O$	10 g.
Ferrous ammonium sulphate	0.7 g.
$MnSO_4.4H_2O$	2 g.
Concentrated HCl	1 ml.
Distilled water to	250 ml.

"Vitamin" Solution C

Aneurine HCl	5 mg.
Pyridoxin	10 mg.
Pyridoxal	5 mg.
Pyridoxamine	5 mg.
p-Amino benzoic acid ...	1 mg.
Riboflavin	5 mg.
Nicotinic acid	5 mg.
Calcium pantothenate ...	5 mg.
Pteroyl glutamic acid ...	5 mg.
Distilled water	20 ml.

This solution is ampouled in 1 ml. amounts and stored in cold.

The complete basal medium (5/3 final strength) consists of:

Solution A	200 ml.
" Vitamin " solution C ...	1 ml. ampoule
Glucose	10 g.
Thiomalic acid	250 mg.
Guanylic acid, Na salt ...	62.5 mg.
Tween 80	0.5 ml.
Sodium phenobarbitone ...	0.5 g.
Biotin, solution of 1 μg./ml. (B.D.H.)	2 ml.
Distilled water to final volume of	300 ml.

The glucose, guanylic acid, and sodium phenobarbitone are dissolved in sufficient water and added to the mixture before the medium is made up to the final volume of 300 ml. This amount of medium is sufficient for the assay of five sera together with a full range of standard B_{12} solutions and a control normal serum.

No pre-treatment of the casein hydrolysate (Allen and Hanbury acid hydrolysed casein for microbiological assay) as carried out by Spray (personal communication) was necessary. By accident, it was also found that adjustment of the *p*H of the basal medium to 5.5 was unnecessary, as the addition of 10 ml. of the cyanide-acetate buffer (*p*H 4.5)-treated solutions brought the *p*H of the final medium to 5.8.

Stock Cultures.—The lactobacillus was first grown in 5 ml. single strength basal medium to which was added 1% soluble proteolysed liver. After overnight incubation and centrifugation the greater part of the supernatant was discarded and the bacterial deposit resuspended in the remaining medium. This was then transferred to sterile ampoules to provide reserve stock cultures. Cultures for use are kept on solid media made from the proteolysed liver medium to which has been added 1% New Zealand agar. Such cultures remain viable for 14 to 21 days at 4° C. The advantage of using a solid medium is that a careful check can be kept on the purity of the strain.

Preparation of the Inoculum.—A single colony from the proteolysed liver agar plate is inoculated into 5 ml. proteolysed liver fluid medium. After overnight (18 hr.) incubation the culture is centrifuged and the supernatant discarded. The bacterial deposit is washed three times in 5 ml. sterile distilled water and finally suspended in 5 ml. sterile distilled water. Then 0.1 ml. of the suspension is inoculated into 5 ml. single strength basal medium and incubated anaerobically overnight (18 hr.), when 0.1 ml. of the undiluted overnight culture is then inoculated into 2 by 5 ml. of the same medium and incubated anaerobically for six hours. The resulting culture provides the inoculum for the assay. Using this technique an even inoculum is obtained with no clumping of the organisms, and there are no irregularities in growth due to the carry-over of adsorbed B_{12}.

Preparation of Serum Extracts and Standard B_{12} Solutions.—To 1 or 2 ml. samples of serum measured with bulb pipettes is added from a burette 19 or 18 ml. of a mixture of NaCN and acetate buffer (0.1% NaCN 20 ml., 0.4 M sodium acetate buffer *p*H 4.5 50 ml., distilled water to 1,000 ml.) and the mixture is autoclaved at 5 lb. for 30 min.

A stock solution of B_{12} is made up by carefully measuring out with a tuberculin syringe 1 ml. of cytamen 50 $\mu\mu$g./ml. (Glaxo) into a litre volumetric flask. This concentrated solution will keep without noticeable deterioration for three months in the ice-chest. From this stock new standard B_{12} solutions containing 25, 50, 100, 200, 400, and 800 $\mu\mu$g./ml. are dispensed for each assay run. Amounts, each of 1 ml., of these standard solutions are then treated in the same manner as the samples of serum. This method of preparing the standard solutions makes for greater accuracy, as a sterile technique is not necessary.

Assay Technique.—Amounts of basal medium, each of 15 ml., are dispensed from a burette into 6 by 1 in. " pyrex " test-tubes, capped with aluminium caps, and autoclaved at 10 lb. for 6 min. To tubes of this medium is then added 10 ml. of either the supernatant obtained from autoclaving the sera under

TABLE I

B_{12} LEVELS OBTAINED IN REPLICATE ASSAYS CARRIED OUT AT INTERVALS USING *LACTOBACILLUS LEISHMANII*

Date	Serum	Replicate Values ($\mu\mu$g./ml.)	Average Value ($\mu\mu$g./ml.)
July, 1956	Normal standard	260 280	*270*
	Patient R.	1,050 1,000	
		950	1,000
Dec., 1956	Normal standard	300 300	*300*
	Patient L.	270 300	285
	,, T.	110 120	115
Jan., 1957	Normal standard	300 330	*315*
	Patient E.	310 350	330
	,, W.	25 40	33
Feb., 1957	Normal standard	275 325	*300*
	Patient Wh.	15 25	20
Mar., 1957	Normal standard	310 310	*310*
	Patient K.	65 65	
May, 1957	Normal standard	300 320	*310*
	Patient P.	100 110	105

Normal standard = 301 ± 21·5.
Normal standard value as determined by an independent laboratory was 310 $\mu\mu$g./ml.

examination, or that obtained from a standard normal serum, or a similarly treated standard B_{12} solution. All the sera are run in duplicate or triplicate. To the now completed medium is added 0.5 ml. of the inoculating culture and the whole shaken well to ensure even mixing. Four 5 ml. samples of the inoculated medium are then transferred (using sterile bulb pipettes) to sterile 6 by $\frac{5}{8}$ in. "pyrex" test-tubes, serially numbered and capped with coloured aluminium caps for identification. Incubation is carried out in a McIntosh and Fildes anaerobic jar simply evacuated with a water-pump and filled with hydrogen. This method of incubation was found to give not only the greatest growth in unit time (36 hr.) but also the most consistent growth in replicate assays. After incubation the density of growth is determined turbidimetrically using a nephalometer (E.E.L.), and from the results obtained with known amounts of B_{12} (25, 50, 100, 200, 400, and 800 $\mu\mu$g./ml.) a standard curve drawn.

Results

Using this technique it has been possible to obtain reproducible results with replicate samples of serum and repeated assays of a control normal serum (Table I).

Discussion

A complete assay, starting from the making of a fresh batch of medium to the obtaining of a final result, can be carried out within a working week. This seems to be of value when it is remembered that the technique described is designed only for the occasional assay. The drawback to occasional determinations, that of irregular results, is eliminated as shown by the results quoted. These determinations all necessitated a completely fresh start.

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AN UNUSUAL CASE OF PROTEINURIA

BY

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Many conditions cause proteinuria, and it has been stated that any patient showing this sign should not be lost sight of until a firm diagnosis has been made. Occasionally the establishment of such a diagnosis is difficult, but the case reported here was finally carried to a successful conclusion.

Case Report

The patient, a married woman of 31, was admitted in October, 1957, complaining of malaise and visual disturbance. She had had a normal pregnancy seven years previously. Two years before admission she said that she had an attack of haematuria, associated with puffiness of the eyes and feet. A diagnosis of acute nephritis was made and she recovered with some residual frequency. She started another pregnancy in July, 1956, and was admitted to another hospital in December with proteinuria and hypertension; a diagnosis of pre-eclampsia was made and labour was induced five weeks prematurely. The baby was healthy and survives, though due to the patient's persistent ill-health she has never been able to look after it. Discharged from hospital in January, 1957, she was readmitted later in the same month for persistent proteinuria and remained in hospital until April. Since discharge she had suffered continual malaise, losing 4 stone in weight and complaining of oliguria and visual disturbances. Physical examination revealed no abnormality apart from a persistent proteinuria. The fundi appeared normal and no lesion could be found to account for the visual disturbance. The patient was extremely disturbed, and for this reason a psychiatric opinion was sought, minimal attention being paid to the proteinuria. An agitated state of depression was diagnosed, possibly psychotic in origin, and treatment for this condition undertaken with E.C.T.

Renal Investigations.—In view of the persistent proteinuria a number of investigations were carried out. The serum urea was 35 mg.% and the creatinine clearance 75 ml./min., which was within normal limits. A proteinuria of 11 to 15 g./day was present, but in spite of this the serum albumin level was normal at 3.2 g.%. This was surprising, for at this level of urinary protein loss, considerable serum protein changes would be expected. In addition the serum cholesterol was normal, and the urine deposit free from formed elements. On paper electrophoresis of serum and urine (Hardwicke, 1954), the urine pattern appeared unusual and when the two specimens were run in parallel on the same strip of paper (Fig. 1) a significant difference in mobility between the two "albumins" was found, while the β globulin seen in the urine had no counterpart in the serum. Immunological examination of the urine against an antibody mixture raised with whole human serum (Gell, 1955) gave a weak reaction for albumin but no reaction for any globulin fraction. This finding was also unusual, serum globulins being present in all ordinary cases of proteinuria. The mean molecular weight of the urine proteins was therefore determined by colloid osmometry (Rowe, 1957); this gave a value of 44,000, a value considerably lower than in any case of proteinuria previously examined, with the possible exception of cases of myelomatosis.

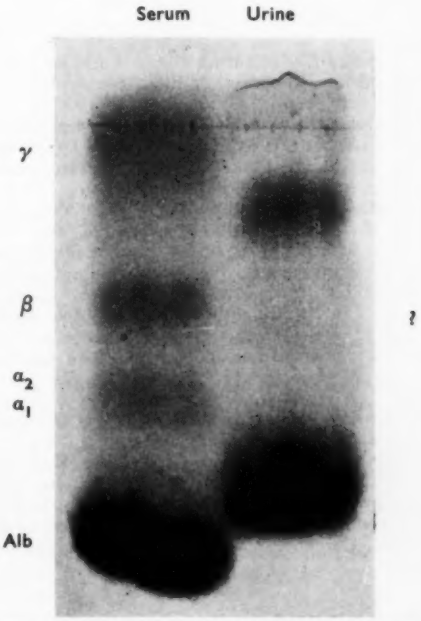

FIG. 1.—Paper electrophoresis of serum and urinary proteins.

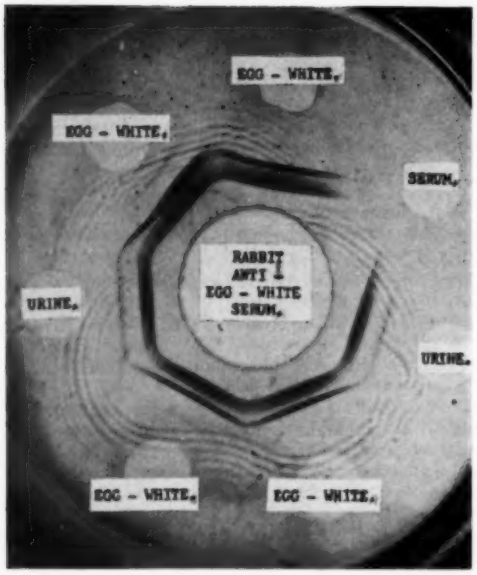

FIG. 2.—Immunological comparison of the patient's serum and urine with whole egg-white. The egg-white and urine specimens show the similar zones of precipitation, representing identical antigens present in both. Serum shows no trace of either of these antigens.

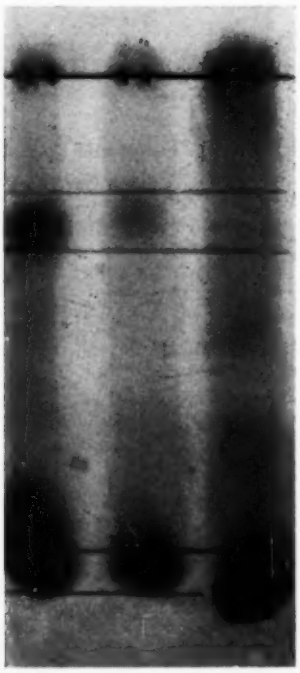

FIG. 3.—Direct comparison of serum, urine, and egg-white by paper electrophoresis.

At this point further specimens of urine were required and a catheter specimen was requested. The ward reported that the urine had been free of protein for the previous two days, and this was confirmed on the specimen obtained. However, a further 24-hour sample proved again to contain over 1 g.% of protein. Close inspection of this sample revealed a thin layer of clear gelatinous sediment which aroused suspicion. Specific precipitation tests were then carried out with rabbit anti-egg-white serum (Fig. 2), and this shows clearly the identity with egg-white of the urine proteins; this finding is further confirmed by comparative electrophoresis (Fig. 3). As a final check a further sample of urine was requested, great emphasis being laid, in the patient's presence, on the interest attached to this specimen. The eggs on the patient's locker were counted and the next day two eggs had disappeared while the 12 g. of protein was in the urine.

Simulated proteinuria is a rare condition in civilian practice and can be difficult to prove. In this case suspicion was aroused by the dissociation of this sign from any other evidence of renal disease. Incontrovertible proof was obtained by immunological techniques, and when challenged the patient admitted the deception, claiming that it was only done to call attention to the kidneys, which she still believed to be diseased. This case appears therefore to fall into the group of "deliberate disability" described by Hawkings, Jones, Sim, and Tibbetts (1956), who consider these problems are unconsciously motivated though consciously executed, and thus differ from true malingering.

I am indebted to the consultant staff of the Queen Elizabeth Hospital for referring the patient for examination, to my colleagues Drs. J. R. Soothill, D. S. Rowe, and D. R. Stanworth for advice and for the application of their specialized technical skills, and to the nursing staff for their co-operation in finally detecting the deception.

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A QUANTITATIVE STUDY OF DECALCIFICATION METHODS IN HISTOLOGY

BY

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The decalcifying of calcified tissues plays an important part in histological technique. However, as it often takes a long time and decreases the staining qualities of the specimen, many attempts have been made to find methods for accelerating this procedure. In this investigation a number of modern decalcifying methods were compared quantitatively.

Methods Described in the Literature

The following methods have been described in the literature.

Heat.—A chemical reaction is accelerated two to three times for every 10° C. rise of temperature, and Murayama, Suzuki, and Itoh (1937) ascertained that with increased temperature the process of decalcification actually takes place within an even shorter time.

Movement.—In the method involving movement the possibility of interaction between the object and the surrounding decalcifying fluid is increased.

Vacuum.—By creating a vacuum the process of decalcification can be characterized as follows:

Insoluble calcium-salts+acid——→soluble calcium-salts+CO_2.

The carbon dioxide, rapidly extracted, will disturb the balance of the chemical equilibrium, and, according to Le Chatelier, result in an acceleration of the reaction. Waerhaug (1949), however, points out that there will be a more intensive contact between the specimen and the surrounding fluid, the CO_2 bubbles being rapidly removed. Waerhaug (1949), Frank and Deluzarche (1950), and Engelbreth-Holm and Plum (1951) also reported a considerable shortening of the time of decalcification. Molenaar (1957) found the decalcification process only slightly accelerated.

Electric Current.—It is supposed that in this procedure, under the influence of an electric field, the Ca ions liberated by the decalcifying fluid will be removed more rapidly. Richman, Gelfand, and Hill (1947), Ducey and Shippy (1950), Dolan (1951), and Scheliga (1952) assert that the process of decalcification is greatly accelerated, whereas the stainability will remain unchanged or even improve, supposedly as the result of the reduced time of action of the decalcifying fluid. According to another group of investigators (Lillie, Laskey, Greco, Burtner, and Jones, 1951), the increased speed of reaction was supposed to result only from the rise in temperature due to the passage of the electric current. These results were confirmed by Molenaar (1957).

As an exact description of the process of decalcification could not be found in the literature, and no mention was made of any fundamental data about the course of this process, there seemed to be good reason for a quantitative approach, the more so as opinions differ on the value of the method of decalcifying with the help of a direct electric current.

Methods

In this investigation the loss of weight was used as an indicator of the rate at which the calcium salts are removed. Specimens were taken from the decalcifying fluid, quickly rinsed, blotted between filter paper, and rapidly weighed with a precision balance accurate to 1 mg.

In a number of experiments the removal of the calcium component was recorded with the help of periodic radiographs.

In a quantitative study it is essential that the test samples should be (1) of equal shape, (2) of equal area, and (3) of equal density.

These requirements are fairly well met by cube-shaped blocks of cortical bone (7 mm. by 7 mm. by 7 mm.), machined as accurately as possible from a horse femur. The weights varied from 690 to 710 mg.

Decalcifying Agents.—The following decalcifying fluids were used.

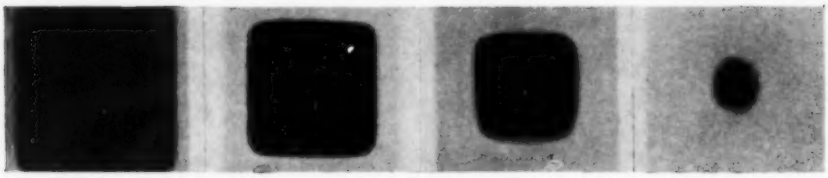

FIG. 1.—Radiographs of different stages of decalcification (about ×4). a, Marginal area of bone block decalcified. b and c, Intermediate stages. d, Last stage, where only the centre of the bone block still contains calcium.

(1) A mixture of equal parts of formic acid 40% and sodium-citrate 20% according to Morse (1945).

(2) Trichloracetic acid 10%.

(3) Nitric acid 5%.

(4) A mixture of hydrochloric acid (38%), 8 volumes, formic acid (85%), 10 volumes, distilled water, 82 volumes, according to Richman *et al.* (1947).

The last-mentioned solution was used for the electrolytic decalcification.

Determination of End-point of Decalcification.—Various methods have been published for ascertaining when complete decalcification has been reached, namely, (1) mechanically by running a sharp needle into the specimen; (2) weighing (Lillie *et al.*, 1951), (3) chemically (Morse), and (4) radiologically (Miles, 1949; Molenaar, 1957).

Miles, by checking radiologically the different stages of the process of decalcification, demonstrated that there is a distinct boundary between the decalcified and the undecalcified area (Fig. 1).

In this investigation it was assumed that the decalcification was complete as soon as the specimen showed no further decrease in weight. (The "time of decalcification" is the time that elapses between the initial stage and the moment that no further decrease in weight can be determined.)

Radiological Determination of Loss of Calcium.—With this method the blocks representing different stages of decalcification were photographed by means of *x* rays and the density of the radiographs measured with Moll's photomicrometer. The results were compared with a standard curve. The latter was determined by making radiographs of a series of specimen blocks with known percentages of loss of weight, and measuring their densities. A Philips *x*-ray apparatus ("oralix") was used. The primary voltage had been lowered from 220 v. to 140 v. so that the tube was run at 30 kv. At this voltage "kodak" F53 film gave radiographs of sufficient contrast for microdensitometry.

In principle this method is certainly suitable, but it requires a complicated measuring apparatus and an elaborate routine, while the results appeared to be less accurate than with the weighing method. By improving this technique, using monochromatic *x* rays, there is a possibility of arriving at quicker and more accurate results. This is beyond the scope of the present investigation, however.

Apparatus.—For experiments at different temperatures an electric oven with a thermostat and flowing tap-water were used.

Experiments *in vacuo* were carried out at a pressure of 20 to 30 mm. Hg, maintained by a jet pump according to Nieuwenhuis (1947).

In a cylindrical glass vessel (diameter 20 cm.; height 20 cm.) containing decalcifying fluid up to a height of 7.5 cm., a specimen block was suspended from a platinum wire reaching beneath the fluid level and moved to and fro (Fig. 2). At first a simple rotary movement of the blocks was tried. This, however, presented the difficulty that the fluid eventually rotated with the blocks.

In a cylindrical glass vessel (diameter 11 cm.) containing 800 ml. decalcifying fluid (Richman, 1947) two platinum electrodes were suspended at a distance of 10 cm. In their immediate neighbourhood

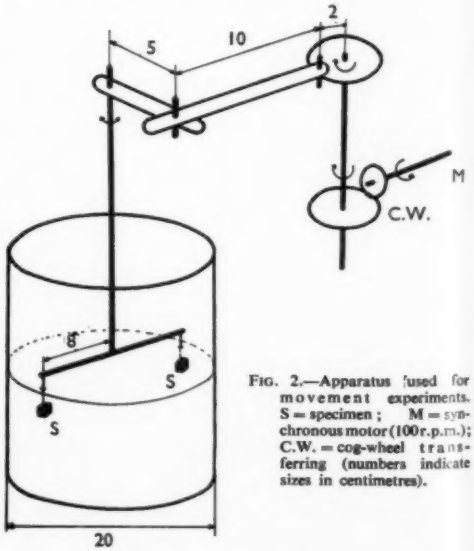

FIG. 2.—Apparatus used for movement experiments. S = specimen; M = synchronous motor (100 r.p.m.); C.W. = cog-wheel transferring (numbers indicate sizes in centimetres).

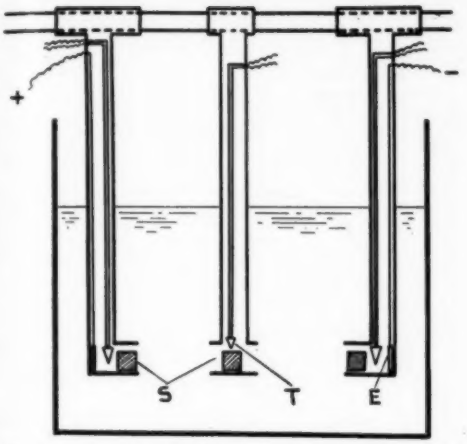

FIG. 3.—Apparatus used for experiments with electric current. S=specimen; E=electrode; T=thermopoint.

two blocks of bone were placed. In addition, halfway between the electrodes an experimental block was suspended. Thermopoints next to all blocks made possible a continuous recording of the locally prevailing temperatures. The passing current (250–300 mA) was obtained by means of a rectifier (Fig. 3).

Presentation of Results.—The results of measurements are presented diagrammatically. Here the concept of the "rate of decalcification" may be introduced, by which is understood the amount of calcium that is extracted per unit of time. In the diagrams the rate of decalcification is indicated by the inclination of the tangent on the curve at that time.

The diagrams are composed of the mean results of at least seven experiments.

Results

Decalcification in Different Acids.—The blocks were freely suspended in the fluids at room temperature (18–20° C.). The decalcification develops most quickly in nitric acid, whereas in trichloric-acetic acid it takes considerably longer. In formic acid decalcification is strikingly slow. Table I gives an instance of the averages of a number of experiments of which Fig. 4 is composed.

Decalcification at Different Temperatures.—Fig. 5 gives the results.

Decalcification in a Vacuum.—Fig. 6 presents the results of this method. Comparison of the curves *in vacuo* with their controls at atmospheric pressure shows that in these experiments the decalcification process was not accelerated at a pressure of 20 to 30 mm. Hg.

TABLE I
TIME OF DECALCIFICATION IN DIFFERENT ACIDS!

Time (Hours)	5% Nitric Acid	10% Trichloracetic Acid	Formic Acid/ Sodium Citrate Mixture
1 hour	10·3%		
3 hours	17·9%	10·7%	
6 "			9·4%
7 "	25·9%		
8 "		17·1%	
24 "	38·9%	27·3%	17·5%
32 "		30·2%	
48 "	44·9%	34·1%	
56 "		35·9%	
3 days	45·8%	38·0%	30·0%
4 "	45·8%	41·2%	
5 "		43·1%	
6 "		44·4%	
7 "		45·0%	39·4%
8 "		45·6%	
9 "			41·5%
12 "		45·7%	43·3%
14 "			43·9%
19 "			44·9%
21 "			45·6%
25 "			45·7%

Example of recording; numbers indicate percentual changes in weight (averages of 10 experiments). Fig. 3 is composed of these data.

Decalcification by Direct Electric Current.—The course of the decalcification process is shown in Fig. 7. The times of decalcification appear to differ greatly, so that with regard to the final results the following data apply to the experiments described:

(1) The following temperatures were recorded during the experiment: anode/cathode 33–36° C.; centre of the vessel: 25–27° C.

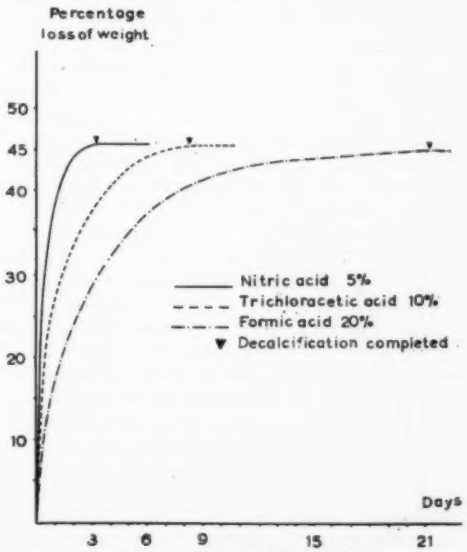

FIG. 4.—Decalcification in various acids.

FIG. 5.—Influence of temperature on the rate of decalcification.

FIG. 6.—Decalcification *in vacuo* compared with decalcification under atmospheric pressure.

FIG. 7.—Rate of electrolytic decalcification as compared with the rate of non-electrolytic decalcification at anode and at room temperature.

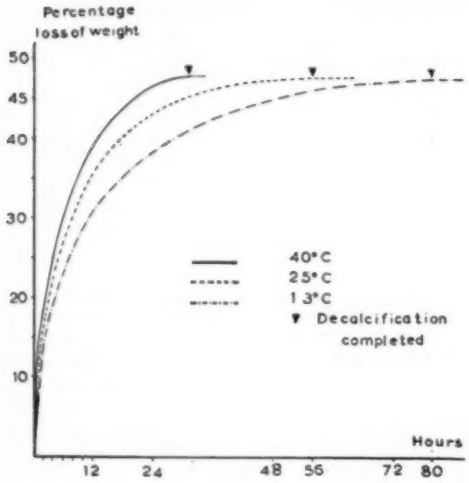

FIG. 5

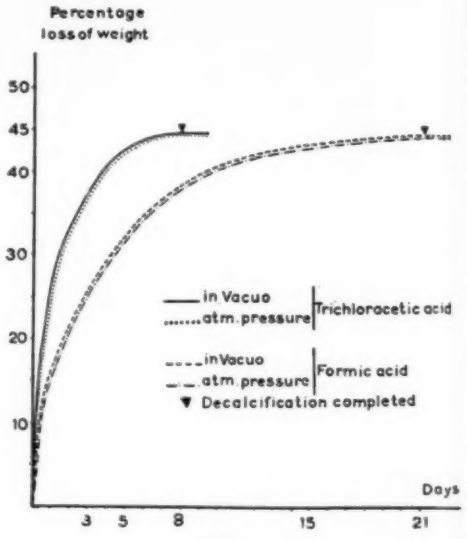

FIG. 6

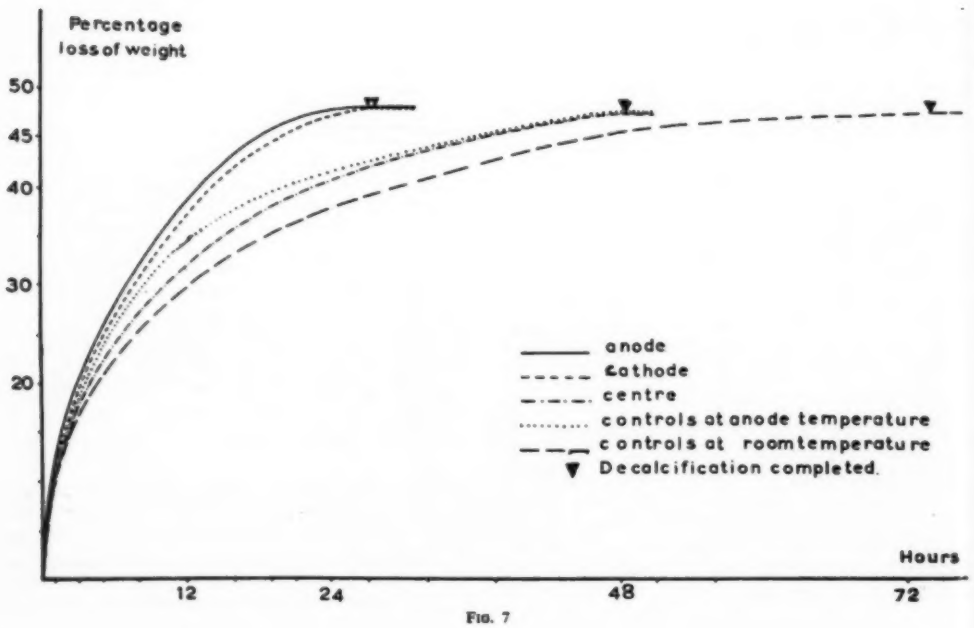

FIG. 7

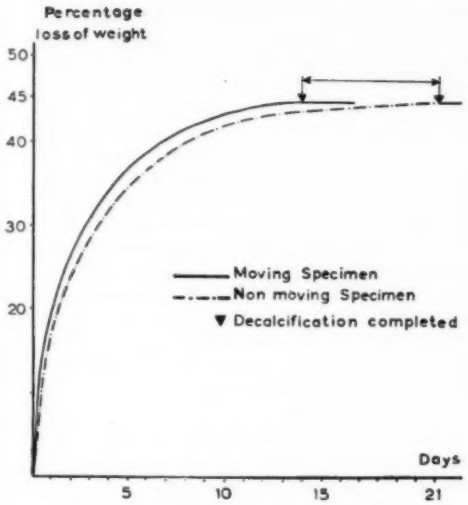

FIG. 8.—Influence of movement (formic acid 20%).

(2) Decalcification on the anode/cathode develops about three times as quickly as with ordinary decalcification.

(3) Decalcification on the anode/cathode develops twice as quickly as in the centre of the electric field.

(4) Decalcification on the anode develops somewhat more rapidly than on the cathode.

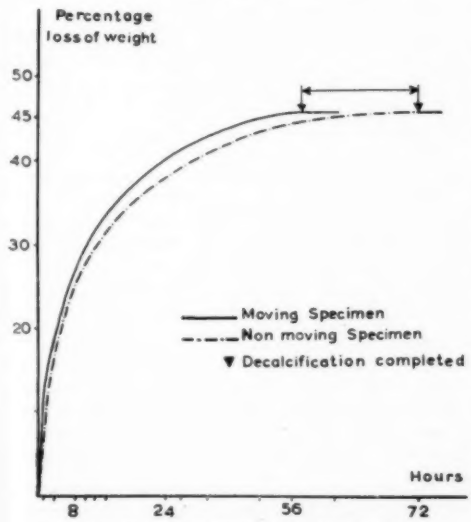

FIG. 9.—Influence of movement (nitric acid 5%).

(5) Decalcification on the anode/cathode develops twice as quickly as in the oven at a temperature equal to that of the anode.

(6) Decalcification of the block in the oven at 35° C. (the temperature at the anode) develops at first more rapidly than that of the block in the centre of the electric field (25–27° C.); in the later stage, when only the centre remains to be decalcified, the reverse takes place, so that both are decalcified in the same time.

Decalcification by Movement. — With this method the results stated in Figs. 8 and 9 are set out. They show clearly that by the reciprocating movement of the samples with respect to the fluid the decalcification process is accelerated considerably. The end-point is reached in two-thirds of the time required by the control block.

Discussion

In calcified tissues the calcium salts are fixed in an organic matrix, which, during the decalcification procedure, keeps its external form. Radiographs of the different stages of the decalcification process show that there is a distinct visible boundary between the decalcified and the non-decalcified part of the specimen block (see Fig. 1). With the exception of the initial stage, therefore, the chemical reaction occurs in the interior of the specimen block.

The "reaction surface," originally the outer surface of the cubical block, gradually takes the spherical form, the surface of which approaches to zero, when decalcification is complete. As the reaction surface decreases in size and the amount of calcium salts extracted per unit of time (= rate of decalcification) is directly proportional to the size of the reaction surface, the rate of decalcification will duly decrease with the development of the process. This factor cannot be influenced. Moreover, the thickness of the layer of organic material between the still calcified part of the specimen and the decalcifying fluid increases, which impedes a free supply and removal of the substances concerned with the reaction. Diffusion also decreases the rate of decalcification in the course of the procedure.

In general it can be concluded that decalcification increases with temperature, but not to such an extent as happens in a purely chemical reaction. These results were as expected. At 40° C. the decalcification process develops about twice as fast as at 13° C. Accordingly, there are theoretically two ways of accelerating the decalcification

process, viz., by increasing the rate of diffusion and the rate of reaction respectively.

In the case of calcified tissues, however, the diffusion factor is apparently so predominant as to make of little use any attempt at influencing the rate of the chemical reaction.

Increasing Rate of Diffusion.—The rate of diffusion may be increased in several different ways :

By Moving the Object.—The principle is to keep the concentration of the molecules and ions of the bone salts on the surface as low as possible ; with the aid of the apparatus described above this diminished concentration is obtained by purely mechanical means, "stripping off" the accumulated particles on the surface ; in addition, the supply of H ions is facilitated.

Lillie *et al.* (1951) found that when the object was stirred continuously in 100 ml. 5% formic acid per gram of bone, the decalcification was only slightly accelerated, for example, the time required was decreased from five to four days (20%). When the object is decalcified in an ample amount of acid (in formic acid as well as in nitric acid), with the aid of the apparatus described here the time required was reduced by about 33%.

Using the Electric Field.—The specific action of the electric current consists in a directed and accelerated movement of the (dissolved) ions when in the orbit of the electric field. Richman (1947) and Scheliga (1952) regard this "forced migration" of the ions as the essential part of the acceleration of the decalcification process. Ducey and Shippy (1950) and Dolan (1951) also found a considerable shortening of the decalcification time, but did not take into account the influence of the raised temperature. Lillie *et al.* deny the specific influence of the electric current and explain the acceleration of the process exclusively by a rise in temperature: the effect is the same when alternating current is used.

The results of our experiments indicate that with this method the rate of decalcification increases not only as a result of the rise in temperature but is also influenced by the electric field. The effect of this current appears from the difference in the rate of decalcification at one and the same temperature with and without passage of a current (Fig. 7).

From a comparison between the curves of a specimen on the anode and that of a specimen at equal temperature in the oven, it appears that the electric field exerts a positive influence, especially in the later stages. The electric field evidently increases the rate of diffusion, and it is the latter that has the most influence on the final stage of the decalcification process and it is the rate of diffusion rather than the rate of reaction which limits the speed of decalcification in its later stages.

In this connexion it is interesting to draw a parallel between the procedure of the decalcification of a specimen between the electrodes (25–27° C.) and a specimen in the oven (37° C.). At first the decalcification of the second specimen develops more rapidly, but later on the reverse is true, in spite of the lower temperature near the first specimen. The slight difference in rate of decalcification between anode and cathode can, in our opinion, be explained by the somewhat lower temperature on the cathode.

By Increasing the Temperature.—By increasing the temperature Lillie *et al.* (1951) found the following differences in time of decalcification (in 100 ml. formic acid 5% per gram of bone) :

At 5° . . . . 7 days
At 23° . . . . 5 days
At 37° . . . . 6 days

Referring to these results these authors observe: "Temperatures of 0–5° C. retard decalcification in aqueous fluid but to a lesser degree than would be expected from the usual effect of changes of temperature on the rate of chemical reactions." They do not raise the question of its cause. Their results cannot be compared with ours because of different experimental series ; however, their statement that decalcification took longer at temperatures of 37° C. than at those of 23° C. is still unaccounted for. ("Decalcification in 100 ml. formic acid 5% per gram of bone at 37° C. actually took longer than at 23° C.")

Murayama and his colleagues (1937), on the other hand, reported with increasing temperature a gradual decrease in the time necessary for decalcification in nitric acid and trichloracetic acid. These last-mentioned findings were in rough agreement with ours (see Fig. 5).

In our opinion the acceleration is caused by an increase in the speed of diffusion of the reaction products rather than by the acceleration of an already very rapid chemical process. If, in the present experiments, the chemical rate of reaction were the only factor, then, with the difference in temperature of 27° C., the decalcification process should not develop two to three times but 12 times as fast.

By Decalcification in a Vacuum.—Another way to speed up the diffusion of one of the reaction

products of the decalcification process is to pump off the gaseous CO_2. Waerhaug (1949) explains the more rapid decalcification he observed by the closer contact between decalcifying fluid and object caused by the rapid removal of the CO_2 gas. Very slight differences in rates of decalcification between the usual method and that *in vacuo* (see Fig. 6) were found in the present series. When the decalcification procedure is watched *in vacuo* it appears that the process is considerably accelerated, mainly due to the "turbulent" development of the CO_2 bubbles. It should be borne in mind, however, that it is the low pressure that causes the considerable expansion of these gas bubbles (at a pressure of 2 cm. Hg the volume increases 38 times compared with that at atmospheric pressure) though the decalcification process is not necessarily accelerated.

According to Le Chatelier the reaction equilibrium is influenced by the dissolution of calcium salts when the formed CO_2 is pumped off rapidly, but we could not perceive any shortening of the decalcification time. In our opinion it is the rapidly expanding gas bubbles that prevent the decalcifying fluid from reaching the surface of the object in an adequate amount, for the same amount of the reaction product (CO_2) occupies a much larger part of the object and "blocks" it from any further supply of fluid. (By "surface" not only the outer surface is understood but also the "inner surface," namely, the surface of the spaces containing the blood vessels as well as that of numerous osteocyte cavities.)

With decrease of pressure the reaction equilibrium is shifted in favour of the rate of decalcification, but the possibility of reaction decreases. Both these factors are supposed to result in fairly equal times of decalcification whether *in vacuo* or not.

Summary

For the purpose of comparing quantitatively some modern methods of decalcification, the loss of weight of identical blocks of cortical bone was determined during the decalcification process.

Acids.—At room temperature the time required for complete decalcification in 5% nitric acid, 10% trichloracetic acid, and a formic-acid-sodium-citrate mixture was three, eight, and 21 days respectively.

Temperatures.—At 13° C., 25° C., and 40° C. the decalcification time in 5% nitric acid took 80, 56, and 32 hours respectively.

Vacuum.—At a decreased atmospheric pressure (to 20 mm. Hg) no acceleration could be demonstrated.

Electric Current.—The decalcification process develops about three times as fast as under normal circumstances. This acceleration is brought about equally by the influence of the electric field and the rise in temperature as a result of the passage of the electric current.

Agitation.—In nitric acid and in a formic-acid-sodium-citrate mixture (according to Morse) the decalcification time was shortened by about 33%.

The loss of nuclear stainability of frozen sections after immersing for varying periods in the decalcifying agents was investigated. Nitric acid 5% was found to be best for teeth and small pieces of bone; for larger pieces of bone a mixture containing equal parts of formic acid 40% and a solution of 20% sodium-citrate (according to Morse) was most suitable both for decalcification and for preserving stainability.

Neither the use of a direct electric current nor the lowering of atmospheric pressure was found to be of any value.

We gratefully recognize the generous and indispensable help during this investigation from H. C. Burger, D.Sc., and A. Noordergraag, M.Sc., of the Department of Medical Physics, Physical Laboratory of the University of Utrecht, and A. J. H. Vendrik, Ph.D., of the Department of Medical Physics, Catholic University of Nijmegen.

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APPENDIX

In general it can be stated that acceleration of the decalcifying process, by whatever method, should never, or, at least as little as possible, decrease the stainability of the specimen. Accordingly the loss of nuclear stainability in frozen sections of formol-fixed tissue of the gums was investigated. The sec-

tions were immersed in the decalcifying fluids as mentioned above for different periods of time, which varied from three hours to three months.

The sections were mounted on coverslips and stained all together with haematoxylin and eosin.

The results are summarized below :

	Start of Perceptible Decrease in Stainability	Marked Decrease in Stainability
Hydrochloric acid (mixture according to Richman)	6 hours	24 hours
Nitric acid 5%	4 days	2 weeks
Trichloracetic acid 10%	3 ,,	1 week
Formic acid/sodium citrate (according to Morse)	2 weeks	2 months

Schajowicz and Cabrini (1955) found that hydrochloric acid and nitric acid rapidly produce important alterations in a number of histochemical stains, whereas with decalcifying fluids containing formic acid the stainability remains unchanged longer. These findings can be related to our results. Decalcification with the aid of the electric current indeed shortens the decalcification time. However, this reduction can only partly, i.e., 50%, be attributed to the influence of the electric current. Moreover, the rapid loss of stainability after immersion in the decalcifying medium according to Richman hardly makes this the method of choice.

Decalcification *in vacuo* has no advantage at all. In the present experiments no measurable acceleration of the decalcification process could be ascertained.

Therefore routine decalcification in this laboratory is carried out as follows :

(1) Small pieces of bone or teeth are decalcified in 5% nitric acid.

(2) Larger pieces are decalcified in a mixture containing equal parts of formic acid 40% and a solution of 20% sodium-citrate (Morse).

(3) Specimens are attached to thin platinum wires and suspended in the stirring apparatus described in this investigation.

J. clin. Path. (1958), **11**, 237.

A CASE OF CUSHING'S SYNDROME WITH PIGMENTATION AND SEVERE HYPOKALAEMIC ALKALOSIS

BY

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From the Friarage Hospital, Northallerton

(RECEIVED FOR PUBLICATION SEPTEMBER 18, 1957)

Hypokalaemia is the most important result of the adrenal hyperfunction seen in the recently described Conn's syndrome (Conn and Louis, 1956). In this syndrome aldosterone is produced excessively, but evidence of the same excess of other hormones is lacking and the excretion of 17-ketosteroids and 17-ketogenic steroids in the urine is normal. Hypokalaemia may also occur in cases with a high urinary excretion of 17-ketosteroids and 17-ketogenic steroids, associated in some cases with the classical clinical features of Cushing's syndrome and in others with few or none of these features (Sprague and Power, 1953; Brooks, McSwiney, Prunty, and Wood, 1957; Foye and Feichtmeir, 1955). In one of these cases excess aldosterone was demonstrated (Brooks *et al.*, 1957) and in another excess mineralo-corticoid (Foye and Feichtmeir, 1955).

The case to be described had some of the features of Cushing's syndrome and presented a hypokalaemic alkalosis more severe than has been described in either Conn's or Cushing's syndrome. In addition there was a melanin pigmentation with a distribution and intensity similar to that seen in Addison's disease. Subtotal adrenalectomy has been effective in reversing all the abnormal features, including the pigmentation.

Case Report

The patient, a married woman aged 42 years, was admitted to the Friarage Hospital, Northallerton, on August 30, 1956. For a year she had felt irritable and depressed but did not become really ill until six weeks before admission. During these six weeks she developed increasing lassitude. She lost nearly a stone in weight and transient swelling of the face and ankles was seen. Increasing pigmentation was first noticed three weeks before admission and about this time there was some dysuria and frequency and a complaint of thirst. Just before admission the patient became slightly confused mentally. Her loss at each menstrual period had been normal, but during the year before admission the intervals between the periods had increased.

On admission she was lethargic and depressed, but showed no gross muscular weakness. There was marked pigmentation of the face, at various pressure sites on the trunk (Fig. 1), on the areolae of the breasts, and on small areas of the mucous membrane inside both cheeks. She was well nourished (10 st. 10 lb.) and showed no abnormal deposits of fat, no kyphosis, and only one small cutaneous stria. The body hair was normal in amount and distribution, but she had a slight, and recent, growth of hair on the upper lip. There was no acne. Blood pressure was 140/80 mm. Hg. Clinical examination revealed no other abnormality. All the deep reflexes were present, and Chvostek's and Trousseau's signs were

FIG. 1.—The patient on admission. The configuration of the face neck, and shoulder does not suggest Cushing's syndrome. Note pigmentation of face, exposed area of neck, and line of pressure from shoulder strap.

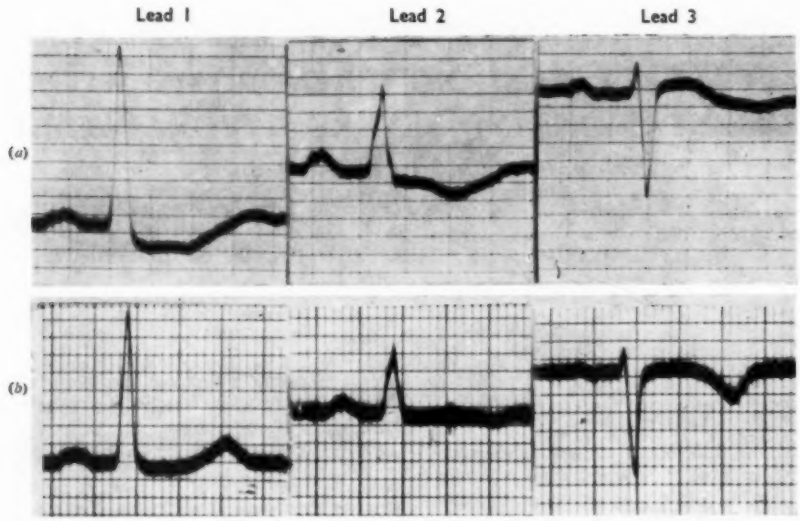

FIG. 2.—Electrocardiographs (*a*) on August 30 showing gross changes typical of potassium deficiency (serum potassium 1.3 mEq./litre); (*b*) on September 2, when standard leads have become normal with the attainment of normal blood levels for potassium (serum potassium 5.6 mEq./litre) although it is highly probable that there was at this time a considerable deficiency of intracellular potassium.

negative. The urine contained sugar and albumin and a moderate number of pus cells and gave a growth of *Bact. coli* on culture.

Addison's disease seemed the probable diagnosis on clinical grounds, but the laboratory findings on the day of admission, far from confirming this, gave results that suggested adrenal hyperfunction, and were as follows: Serum potassium, 1.3 mEq./litre, serum sodium, 148 mEq./litre, serum chloride, 83 mEq./litre, serum bicarbonate, 52 mEq./litre. Leucocytes, 11,000 per c.mm., blood sugar, 210 mg.%, blood urea, 27 mg.%, Hb 75%. An E.C.G. showed changes in keeping with gross potassium deficiency (Fig. 2).

Before investigating the case further, measures were at once taken to correct the hypokalaemia. Potassium chloride by mouth, 2 g. two-hourly, was started at 11 p.m. on the day of admission. Next day the patient was very much brighter and the serum potassium level was 2 mEq./litre. Treatment with even larger doses of potassium chloride continued and within four days the serum potassium had returned to normal. Within the same period, without any administration of ammonium chloride, the severe alkalosis observed on admission disappeared and the plasma bicarbonate and chloride levels became normal.

A glucose tolerance test gave a diabetic type of curve. There were no circulating eosinophils, and the excretion of 17-ketosteroids was 36 mg. in 24 hours. Professor F. T. G. Prunty then arranged for the estimation of the output of other steroids, including

TABLE

STEROID EXCRETION AND A.C.T.H. RESPONSE TEST[1]

Date	Potassium Supplement (mEq./24 hr.)	Dose of A.C.T.H. (units/24 hr.)	17-Keto-steroids (mg./24 hr.)	17-Ketogenic Steroids (mg./24 hr.)	Aldosterone (mg./24 hr.)[2]
Pre-operative					
4/9/56	Nil	Nil	30·0	—	—
5/9/56	445	,,	35·4	90	1
12/9/56	Nil	,,	34·2	114	—
13/9/56	,,	,,	24·8	109	2
14/9/56	,,	40	43·3	126	—
15/9/56	,,	40	43·7	68	—
16/9/56	,,	40	37·6	100	—
17/9/56	161	40	37·0	118	—
Post-operative					
21/11/56	Patient on 0·5 mg. 9α fluorohydrocortisone daily (no metabolites appear in urine)		6·8	0·5	

OTHER URINARY STEROIDS

Chromatography[3] (Bush C system) of glucuronidase-hydrolysed urine extract indicated:

Tetrahydrocortisone = 5 mg./24 hr.
Tetrahydrohydrocortisone = 15 mg./24 hr.[4]
Free cortisone = 4 mg./24 hr.
Total 24 mg., which is greatly in excess of normal.

PRE-OPERATIVE PLASMA LEVELS

Intermedin[5] (M.S.H.) .. None found.
A.C.T.H.[6] .. Significant amounts (more than normal).
Hydrocortisone .. 12 μg./100 ml. (upper limit of normal 8 μg., mean normal 5 μg.).
Corticosterone .. Quantity present could not have exceeded 3 μg./100 ml.: none found.

[1] Dr. B. W. Brooks. [2] Dr. I. H. Mills. [3] Bush (1952). [4] No standard was available as a check on identity. [5] Kindly carried out by Professor F. W. Landgrebe. [6] Dr. Barbara E. Clayton.

aldosterone, and for the measurement of the effect on the 17-ketosteroid and 17-ketogenic steroid output on giving A.C.T.H. (Prunty, 1956). Professor Prunty supplied a batch of A.C.T.H. of known potency for this purpose, and at his suggestion potassium supplements were stopped during the period of the test to exclude any secondary effect on the aldosterone output from the administration of potassium in large quantities.

The results (Table) were in keeping with Cushing's syndrome. It was thought that the lack of significant response of the steroid excretion to A.C.T.H. was due to a tumour. On admission the only classical clinical features of Cushing's syndrome had been the presence of a little hair on the upper lip, but within a week this had noticeably increased, and after a further fortnight there was an obviously abnormal degree of facial hirsutism. At the same time, marked acne appeared. The blood pressure, however, always remained normal.

Metabolic Studies

Meanwhile the study of the potassium and sodium metabolism continued. The time before operation divides into two periods (periods 1 and 3) when the patient was receiving a large potassium supplement; and a middle period (2) when all extra potassium was omitted to facilitate the study of the steroid excretion (Fig. 3*a*). During periods 1 and 3 the serum potassium, initially at a low level, rapidly rose to normal when a large potassium supplement was given, only to fall equally rapidly when it was omittted. During this time a large proportion of the potassium supplement was lost in the urine, but it is clear that some was retained. (During period 1, the average potassium supplement was 254 mEq. a day and the average urine output 201 mEq. a day; during period 3, the supplement was 278 mEq. and the urine output 194 mEq.). The difficulty experienced by the patient in retaining potassium was illustrated in period 2. No supplement was given and the serum potassium at once fell to 2 mEq./litre and remained at this level throughout the six-day period, yet the patient continued to excrete potassium at an average rate of 55 mEq. per day. At the same time her clinical condition deteriorated and on several occasions her legs gave way.

The serum sodium was always normal, but during period 2 it is probable that the patient was retaining sodium since the average excretion on a normal ward diet was only 44 mEq. per day. During periods 1 and 3, the average excretion of sodium was at a higher level (148 mEq. per day in period 1). In fact during the pre-operative period there was a rough parallel between the sodium and the potassium excretion. When the excretion of potassium rose as the result of the potassium supplement so also did the excretion of sodium. This effect was not seen after adrenalectomy.

Studies were also made of the sodium/potassium ratio in saliva and sweat. The results will be referred to in the discussion.

Towards the end of the first month it had become clear that an operation would be necessary. The A.C.T.H. response test suggested that the patient had an adrenal tumour, but tomograms taken after the presacral insufflation of oxygen clearly demonstrated the outlines of the adrenals and there seemed to be no tumour. An operation for the one-stage removal of the adrenals in cases of carcinoma of the breast through a semilunar incision placed transversely in the epigastrium (Fig. 6) had recently been developed here (McKeown and Ganguli, 1956). This operation allowed both adrenals to be inspected before action was taken, and meant that a pre-operative differential diagnosis between tumour and hyperplasia became less important. The operation was carried out, with Dr. K. H. Oldfield as the anaesthetist, on October 1.

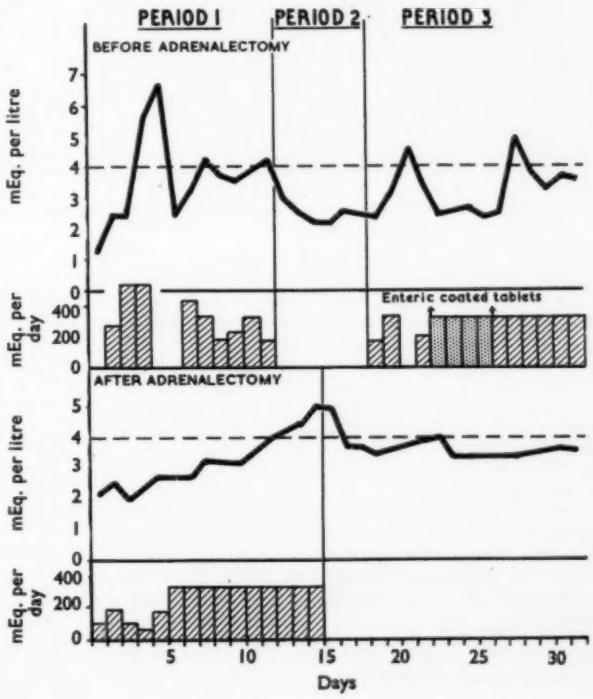

FIG. 3.—Diagram showing daily potassium supplements and serum potassium levels (*a*) before adrenalectomy, (*b*) following adrenalectomy. Note that early in period 3 potassium was given for four days as enteric-coated tablets in an effort to avoid gastric irritation, but was incompletely absorbed.

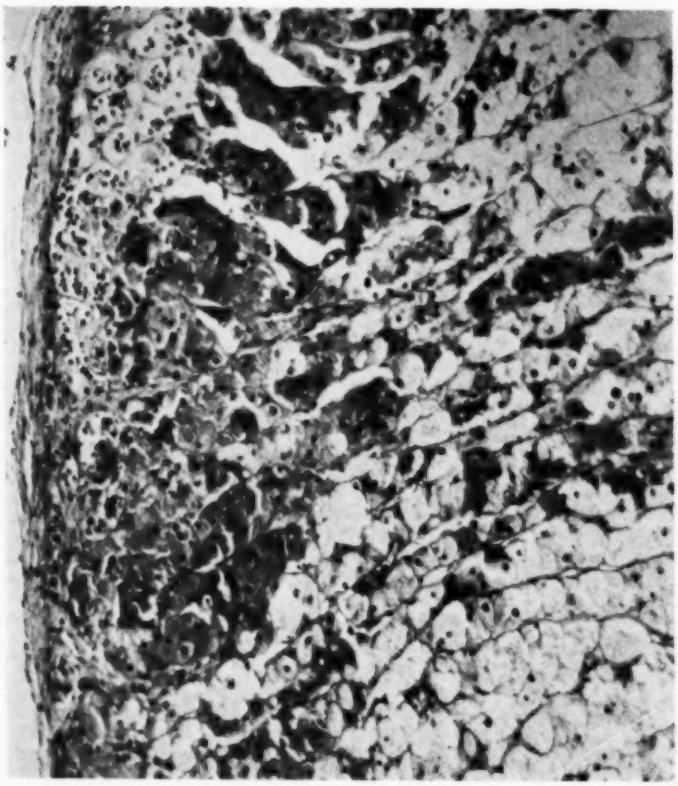

FIG. 4.—Section through the left adrenal cortex. An island of the poorly developed zona glomerulosa is seen, top left; elsewhere the zona fasciculata reaches the capsule. (Haematoxylin and eosin, × 150.)

There was no tumour but both adrenals were hyperplastic. The whole of the left, and nine-tenths of the right, adrenal were removed. Biopsies were taken from the kidney, from the liver (because it looked abnormal), and from the rectus muscle.

The left adrenal weighed 15 g. and the right 10 g. Section showed a very considerable hyperplasia of the zona fasciculata of the cortex, but the glomerulosa was thin and poorly developed, being replaced by the fasciculata beneath the capsule in many places (Fig. 4). Frozen sections showed abundant anisotropic lipoid (Fig. 5).

Sections from the kidney biopsy showed in some degree the vacuolation of the first convoluted tubules that has been noticed in hypokalaemia (Chalmers, Fitzgerald, James, and Scarborough, 1956). The liver biopsy showed a very considerable degree of fatty infiltration.

The patient had a stormy and then difficult convalescence, but within four months her strength had returned, pigmentation had disappeared (Fig. 6), and

FIG. 5.—Frozen section of the left adrenal viewed through partially crossed polaroid screens. There is abundant anisotropic lipoid somewhat irregularly distributed (× 7.5).

FIG. 6.—The patient five months after adrenalectomy; pigmentation has gone. The transverse scar of the operation is shown.

she was able to lead a normal life. The only disappointment has been that, in spite of efforts to leave sufficient adrenal tissue for her needs, it has not been possible to dispense with a daily maintenance dose of 37.5 mg. cortisone.

The very considerable deficit of potassium that existed at the time of operation was shown by the analysis of the muscle biopsy carried out by Mr. F. Harper, Central Clinical Laboratory, Middlesbrough. The potassium and sodium content were 34 and 51 mEq. per kg. fresh muscle compared with average normal values of 76 and 30 mEq. per kg. respectively (10 observations on normal muscle; for potassium, range was 51–104, standard deviation 18; for sodium, range 22–42, standard deviation 7.5). Some time elapsed after the operation before this deficit was corrected. Immediately after the operation the serum potassium had fallen to 2.2 mEq./litre, and, as soon as the patient could take it, the same high potassium supplement was again given. This time the serum level rose slowly, but when the normal level was attained on the 15th post-operative day no further potassium supplements were required to sustain it (Fig. 2b). In spite of the slow rise of the serum potassium, the urine excretion rate showed that a greater proportion of the supplement than before was being retained. (Before operation, on a daily average supplement of 254 mEq. the urine output was 201 mEq.; after operation on a supplement of 245 mEq. the output was 101 mEq.)

Discussion

The case has special interest because of the two dominant features, hypokalaemia and pigmentation. It was also interesting that a number of the clinical features of Cushing's syndrome were missing.

When the patient was first seen the only classical feature of Cushing's syndrome shown was a little hair on the upper lip. Possibly the rapidity of onset had left insufficient time for the other changes to occur; certainly the speed with which in the weeks following admission the growth of the facial hair increased and acne developed suggested a rapidly progressive disease. The blood pressure was always normal. Hypertension is absent in a proportion of cases of Cushing's syndrome (Cope and Raker, 1955), but has been invariable in all cases of primary aldosteronism so far described. In view of the close similarity of the electrolyte picture in the present case to that seen in aldosteronism, it seems surprising that there was no hypertension.

The Electrolyte Disturbance.—The pattern of the electrolyte disturbances was exactly similar both qualitatively and quantitatively to that observed in cases of Conn's syndrome. One of the features, well illustrated in the present case, has been the subject of comment by Prunty and his colleagues (Ager, McSwiney, and Prunty, 1956). They draw attention to the rapid rise to normal of the serum level that is seen on administration of potassium in cases of hypokalaemia due to endocrine dysfunction. This is contrasted with the slower rise seen in potassium depletion due to other causes (chronic diarrhoea, pyelonephritis). The authors consider that the difference suggests a decreased transfer of potassium to the intracellular pool in the former group. In this patient the period immediately following operation, when the serum potassium rose but slowly in spite of large supplements, can be compared to the latter type of case since at this time the endocrine dysfunction had been corrected by adrenalectomy but the patient was depleted of potassium.

An abnormally high secretion of potassium in saliva and sweat is among the recognized effects of primary aldosteronism (Milne and Muehrcke, 1956). Abnormal sodium and potassium ratios were observed in the present case and corresponded very closely with the findings described in cases of aldosteronism. Observations on the Na/K ratio in the saliva on a number of occasions before operation were compared with observations made on saliva from normal subjects and on the patient after adrenalectomy. As has been found by other workers (Pawan, 1955; Eales and Linder, 1956), the wide variations in the results obtained on different occasions both in the patient

and in normal subjects limited the value of observations on saliva as a method of demonstrating endocrine dysfunction. Nevertheless it was possible to demonstrate that both the mean values and the range of the salivary Na/K ratios in the patient before adrenalectomy were low compared with normal subjects and returned to normal following adrenalectomy. The results obtained were as follows (10 observations in each series): Before adrenalectomy the mean Na/K ratio was 0.27 with a range of 0.11 to 0.37, standard deviation 0.13, as compared with a mean for normal subjects of 0.36, range 0.25 to 0.47, standard deviation 0.06. After adrenalectomy the mean value for the patient was 0.35, range 0.26 to 0.51, standard deviation 0.06. (A simple technique for the determination of the Na/K ratio in sweat was employed, the sweat being collected on a filter paper held in the axilla.) Two observations gave Na/K ratios of 1 compared with normal values of 13–16 (Cantarow and Trumper, 1955). After adrenalectomy it was not possible to obtain a satisfactory measurement of the concentration of potassium by the simple technique in use.

It is of interest that, despite the close similarity between the electrolyte picture in the present case with that of primary aldosteronism, no excessive secretion of aldosterone was found in the urine. The unreliability of present methods of aldosterone estimation must, however, be recognized and normal figures have been found in Conn's syndrome (Evans and Milne, 1954; Russell, Marshall, and Stanton, 1956). The question remains, therefore, whether the electrolyte changes were produced by aldosterone or by another steroid with mineralo-corticoid effect, possibly corticosterone.

It was thought that the histological appearance of the adrenals could throw light on this problem. There is evidence that in the normal adrenal aldosterone is formed in the glomerulosa (Bartter, 1956), and in the present case the appearance of the glomerulosa did not suggest hyperfunction, rather the reverse. It is possible, however, that in the abnormal adrenal aldosterone is also produced in the fasciculata. This is the opinion of van Buchem, Doorenbos, and Elings (1956), who have described the only case so far of primary aldosteronism in which over-production of hormone was due not to tumour but to hyperplasia of the glands. By the courtesy of Dr. Arends and Professor van Buchem the sections from their patient have been compared with those of our own. Their sections differ in that the zona glomerulosa can everywhere be readily made out, and in some few places is hyperplastic, but in the sections from both cases a markedly hyperplastic zona fasciculata dominates the picture.

Pigmentation.—Pigmentation is an occasional feature of Cushing's syndrome. Simpson (1956) mentions pigmentation of the orbit and nipples, and Cope and Raker (1955) remark on it in a few of their cases but do not mention its extent. In the present case the intensity and distribution of the pigmentation resembled that seen in Addison's disease.

Pigmentation in Addison's disease is now thought to be due to excessive production of melanocyte-stimulating hormone by an overactive pituitary. Sulman (1952, 1956) was able to demonstrate excess in nearly all the samples of plasma from cases of Addison's disease examined. He also found excess in the plasma in 30 out of 45 cases of Cushing's syndrome. Sulman claims that excess of melanocyte-stimulating hormone is always accompanied by excess of corticotropin and considers that by demonstrating an excess of melanocyte-stimulating hormone it is possible to distinguish the "pituitary" from the purely "adrenal" type of Cushing's syndrome. Geschwind, Li, and Barnafi (1956) have reported the isolation of pure melanocyte-stimulating hormone and find that it contains seven amino-acids which occur in an identical sequence in corticotropin. They suggest that this explains the melanocyte-stimulating activity of pure corticotropin.

It would seem probable, therefore, that the pigmentation in the present case represented pituitary overactivity. Excess A.C.T.H. was found in the plasma and may have itself been the stimulus for the pigmentation, since no excess of melanocyte-stimulating hormone was found in the single sample of plasma examined. It is also probable that pituitary overactivity was the reason for the failure of the urinary steroid excretion to respond to test injections of A.C.T.H. The adrenals were already responding maximally to endogenous A.C.T.H., and could do no more. An example of a similar failure of the steroid excretion to respond to A.C.T.H. in a case of adrenal hyperfunction not due to adrenal tumour has been reported by Rosenthal (1957). In this case also there was a melanin pigmentation with a distribution characteristic of Addison's disease.

The disappearance of the pigmentation after adrenalectomy calls for an explanation, the more so since many cases of Cushing's syndrome develop pigmentation after adrenalectomy (Sprague, Kvale, and Priestley, 1953; Hernberg, 1954). The answer may derive from the fact that in some respects the actions of cortisone and aldosterone

are antagonistic (Hetzel, McSwiney, Mills, and Prunty, 1956; Rosenbaum, Papper, and Ashley, 1955). It may be that before the operation the pituitary was overacting in an attempt to stimulate production of more cortisone to counteract the excessive mineralo-corticoid effect from other hormones. When the latter was removed by operation this stimulus to A.C.T.H. production would disappear.

Summary

A woman of 42 years with adrenal hyperplasia had pigmentation resembling that of Addison's disease, normal blood pressure, and severe hypokalaemic alkalosis. Subtotal adrenalectomy led to the restoration of normal blood chemistry and disappearance of the abnormal pigmentation.

Our thanks are due to Professor F. T. G. Prunty for his generous help and advice in the diagnosis of the case and in the preparation of this paper. We wish to thank also, in addition to those mentioned in the text, Dr. E. Gilbert for referring this patient, Dr. Simon Sevitt for his opinion on the histology, Sister Calvert, Sister Gibson, and Dr. E. R. L. Paton, of the Friarage Hospital, for their tireless efforts in caring for this patient, Mr. S. Taylor for the photography, and Mrs. R. Carver for the biochemical estimations.

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GIANT CELL GRANULOMATA IN REGIONAL LYMPH NODES OF CARCINOMA

BY

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The occurrence of sarcoid-like lesions within neoplasms and in regional lymph nodes is now a well recognized phenomenon (Marshall, 1956). Examples of such lesions have been reported in association with a variety of tumours, for example, carcinoma of breast, skin, bronchus, and bile ducts, ovarian tumours, and neurocytoma (Refvem, 1954).

The association of generalized sarcoidosis with carcinoma is decidedly rare. Gherardi (1950) presented a case and was unable to find any other example in the literature up to that date: Symmers (1951) questions the authenticity of generalized sarcoidosis in Gherardi's case, as the necropsy was of limited extent.

The object of this paper is to present a case of carcinoma associated with generalized sarcoid lesions in lymph nodes and 10 cases of carcinoma in which the regional lymph nodes show diverse granulomata and giant cell reactions.

Material and Methods

The tissues examined were from surgical and necropsy material. Fixation was by formal saline, Heidenhain's Susa, or corrosive formal. All tissues showing giant cell lesions were stained by haematoxylin and eosin, Gordon-Sweets' method for reticulin fibres, Weigert's resorcin fuchsin and Ponceau S for elastic tissue and collagen, and by Perls' method for haemosiderin.

Case Reports

Case 1 (P53/425).—T. H., a man aged 53, had a carcinoma of the stomach with generalized "sarcoid lesions."

This man had suffered epigastric pain after meals for five years before a gastro-enterostomy was done in 1941. His symptoms largely disappeared, but pain recurred in 1952. Vomiting occasionally accompanied the pain, but became more severe, and he was admitted to hospital for investigation in December, 1953.

Examination showed a rather thin, pale man; there was some upper abdominal tenderness but no other important abnormality. The blood haemoglobin was 8.6 g. per 100 ml.; a barium meal showed a functioning gastro-enterostomy but no evidence of ulcer.

At laparotomy a large pyloric mass was found and a total gastrectomy was done. Post-operative convalescence was uneventful until the tenth day when he suddenly collapsed and died.

A massive intraperitoneal haemorrhage was found at necropsy; it arose from the site of oesophageal and jejunal anastomosis, although the vessel concerned was not identified. No evidence of metastasis was found, the only other abnormality being enlargement of the spleen (615 g.) and slight enlargement of lymph nodes in the neck, mediastinum, and abdomen.

Histology.—The surgical specimen was an acinar columnar cell carcinoma. Two lymph nodes from the lesser omentum contained metastases; one of these contained sarcoid-like lesions which were also seen in another node not involved by growth. The sarcoid-like lesions (Fig. 1) consisted of numerous discrete, rounded areas of eosinophilic histiocytes, the latter having ill-defined outlines; most of the cells were surrounded by a reticulin strand. Giant cells containing up to 40 nuclei, which were often peripheral in position, were found within the areas of histiocytes. Many giant cells contained clusters of globular inclusions (Fig. 2) and transitions between these and asteroid bodies could be found.

Sections of lymph nodes, lung, and liver, taken after death, showed histiocytes and giant cells mingled together with no follicular arrangement. The lymph nodes were diffusely involved by this process (Fig. 3) and many giant cells contained asteroids and globular bodies. There was some slight fibrosis throughout the node and each histiocyte was encircled with a reticulin strand. The appearances agree with those considered by Robb-Smith (1938) and Cowdell (1954) to be characteristic of Stengel-Wolbach sclerosis. The sections of spleen showed no evidence of granulomatous lesions.

Case 2 (P54/025).—H. P., a woman aged 63, had a carcinoma of the thyroid and Stengel-Wolbach sclerosis.

This woman was admitted to hospital in December, 1953, for the investigation of low back pain which

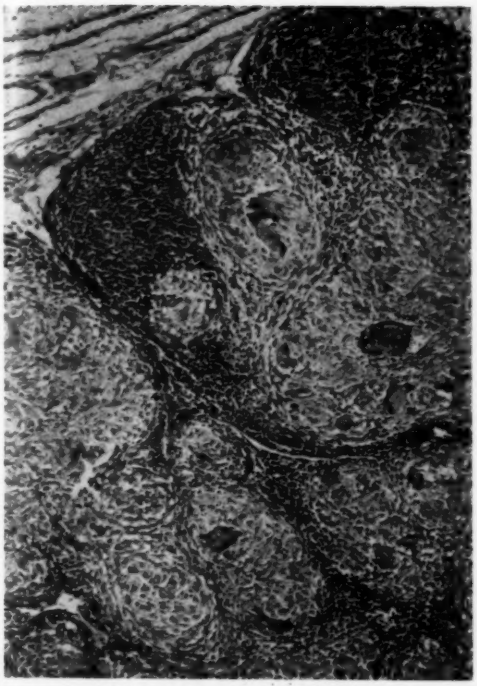

FIG. 1.—Sarcoid-like lesions in a lymph node from the lesser omentum (haematoxylin and eosin × 90).

had been present for two months. On examination she was pale and a firm mass of lymph nodes was found in the left supraclavicular triangle. The blood haemoglobin was 11.4 g. per 100 ml.; no occult blood was detected in the faeces. A biopsy of the enlarged nodes showed that they were largely replaced by a polygonal cell carcinoma the source of which remained uncertain. She deteriorated rapidly following the biopsy and died.

Necropsy revealed a spherical white mass (2 cm. diameter) in the right lobe of the thyroid gland. A few small metastases were found in the lower lobes of both lungs; each pleural sac contained about 200 ml. of blood-stained fluid. Metastases were seen in the cut surface of the right femur, vertebrae, and sternum.

Cervical, mediastinal, coeliac, and other abdominal lymph nodes contained growth. One node alongside the abdominal aorta was enlarged but did not contain macroscopic growth. Nodules of growth were present in the liver. The spleen was rather small (155 g.) and had a purple-brown cut surface in which were slightly raised, rather ill-defined areas (approximately 1.5 cm. diameter).

No important abnormality was found in any other organ.

Histology.—The thyroid nodule was a polygonal cell carcinoma; similar tumour tissue was found in sections of the liver, lungs, vertebrae, sternum, femur, and cervical and coeliac lymph nodes. Numerous rounded follicles, composed of histiocytes with ill-defined boundaries, were scattered throughout the section of spleen. Most of the follicles contained giant cells with globular inclusions; a few had asteroid inclusions (Fig. 4). Each follicle was surrounded by a delicate reticulin network and reticulin fibres passed radially into the follicles enclosing each histiocyte in a single strand. Some histiocytes contained haemosiderin and many of the follicles lay adjacent to clumps of haemosiderin pigment.

The para-aortic node contained scattered giant cells with globular or, less often, asteroid inclusions (Fig. 5). Very occasionally a giant cell showed anisotropic material. Follicles of histiocytes were not seen. A few sinuses were replaced by hyalinized fibrous tissue.

Case 3 (S49/2093).—A. H., a man aged 65, had a carcinoma of the tongue and a giant cell reaction in the cervical lymph nodes.

This man was first seen in May, 1949, with a small carcinomatous ulcer on the right side of the tongue

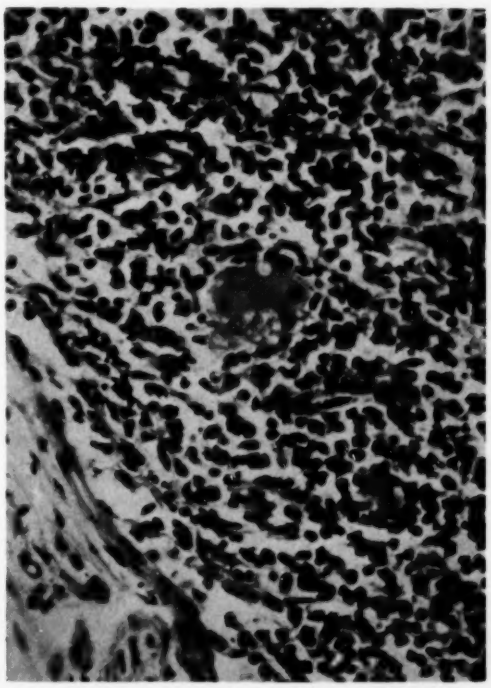

FIG. 2.—Globular inclusions in a giant cell (haematoxylin and eosin × 350).

which was treated by irradiation. The response was not satisfactory and he was readmitted in August, 1949, when the carcinoma was excised and a block dissection of right cervical lymph nodes was done. Recovery has been uneventful and there is no evidence of recurrence.

Histology.—The tumour was a well differentiated squamous carcinoma. Three cervical lymph nodes contained growth; in one of these clusters of giant cells, two of which contained globular inclusions, were present near the peripheral sinus. Rounded follicles of histiocytes were not conspicuous, though two areas in the node contained groups of histiocytes bounded by connective tissue suggesting a follicular arrangement. Reticulin fibres did not surround the histiocytes or the follicles themselves.

Case 4 (S54/4102).—A man aged 59 had a carcinoma of the stomach and sarcoid-like lesions in the omental lymph nodes.

This man was admitted to hospital in November, 1954, for the investigation of epigastric pain. A laparotomy was done and showed a carcinoma encircling the stomach about midway along its curvatures. A total gastrectomy was performed.

FIG. 3.—Diffuse mixture of histiocytes and giant cells in a lymph node (haematoxylin and eosin × 35).

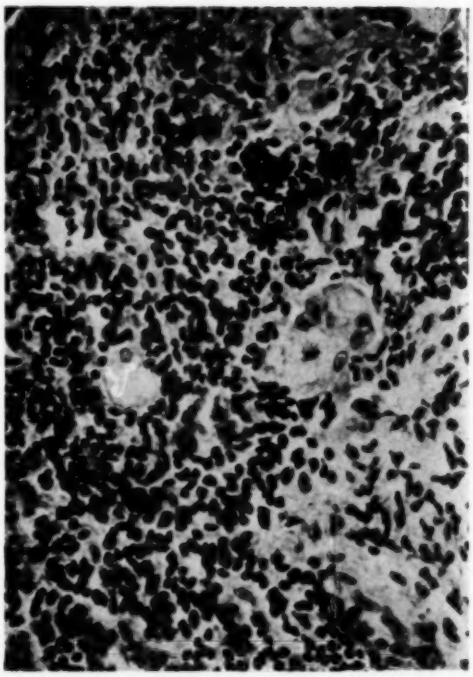

FIG. 4.—Asteroid and globular inclusions in a giant cell (haematoxylin and eosin × 350).

Malignant ascites developed in August, 1955, the liver became enlarged, due to metastases, and he died. A necropsy was refused.

Histology.—The gastric tumour was a tubular cubical cell carcinoma. None of the lymph nodes sectioned contained growth; however, two from the lesser omentum contained peripheral follicles of histiocytes occasionally situated in the peripheral lymph sinus (Fig. 6). These follicles were composed of histiocytes with poorly defined boundaries; fibrosis within or around the follicles was not a conspicuous feature, nor were reticulin fibres detected in or around them. Occasional giant cells without inclusions were found in the follicles.

Case 5 (S54/3238).—W. B., a man aged 50, had a malignant melanoma of the foot and "Stengel-Wolbach lesions" in the inguinal lymph nodes.

This man was admitted to hospital in September, 1954, for removal of a melanoma of the right foot; a block dissection of the inguinal lymph nodes was done at the same time. He had not shown any clinical evidence of recurrence when last seen in February, 1956.

Histology.—The lesion on the foot was a malignant melanoma. Three of the 10 inguinal lymph nodes

contained giant cells, with globular and asteroid inclusions, mingled with histiocytes which showed clearly defined cytoplasmic boundaries. The histiocytes were not grouped into a follicular arrangement, but these and the giant cells were surrounded by a strand of reticulin. Clumps of haemosiderin pigment were seen in the areas of giant cells. Fibrosis was not a conspicuous feature of these nodes. One of the remaining nodes contained a deposit of a malignant melanoma.

Case 6 (P54/403).—W. C., a man aged 54, had a carcinoma of the stomach with a giant cell reaction in omental lymph nodes containing growth.

This man was admitted to hospital in August, 1954, with a two-month history of pain and fullness in the epigastrium. An upper abdominal mass was felt on examination and a barium meal confirmed the clinical diagnosis of carcinoma of the stomach.

A total gastrectomy and splenectomy was done in September, 1954. He developed peritonitis and died nine days later.

Necropsy confirmed the diagnosis of peritonitis due to a leak from the oesophago-jejunal anastomosis; a left lower lobe bronchopneumonia was also present. No evidence of metastatic carcinoma was found at necropsy.

Histology.—The gastric tumour was a spheroidal cell mucus-producing carcinoma. Four lymph nodes taken from the lesser omentum were diffusely infiltrated by carcinoma cells; mingled with the tumour cells were giant cells, some of which contained globular bodies (Fig. 7). The latter bodies did not stain pink with mucicarmine. Lymph nodes were examined, at necropsy, from the cervical, mediastinal, abdominal, inguinal, and axillary groups; none of these contained growth or giant cells.

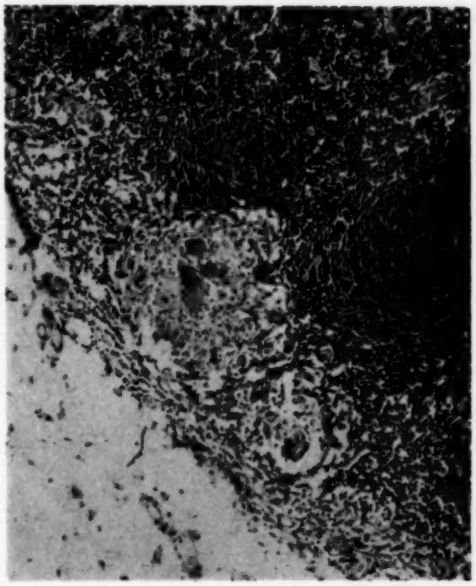

FIG. 6.—Peripheral lesions in a lymph node (haematoxylin and eosin × 90).

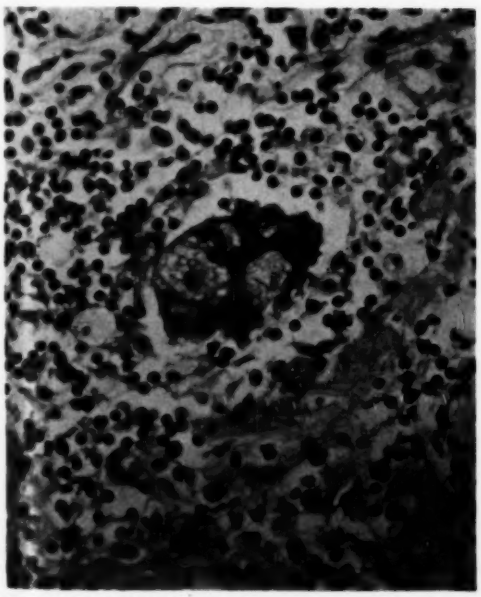

FIG. 5.—Globular inclusions in a giant cell (haematoxylin and eosin × 350).

Case 7 (S53/4551).—E. K., a woman aged 55, had a carcinoma of the stomach and a giant cell reaction in the neoplasm and in an omental lymph node.

This woman was admitted to hospital in October, 1953, for the investigation of epigastric pain. A carcinoma of the stomach was found at laparotomy and removed by a total gastrectomy. Convalescence was uneventful.

The liver was found to be enlarged and nodular in March, 1955. She was admitted to hospital in April, 1955, because of acute intestinal obstruction and died in a few hours. Necropsy was refused.

Histology.—The gastric neoplasm was a diffusely infiltrating carcinoma. Foci of histiocytes with poorly defined boundaries were found in the circular muscle coat adjacent to the carcinoma (Fig. 8). A few giant cells were found in the follicles, but did not contain inclusions; a small amount of anisotropic material was found related to some of the giant cells. Two lymph nodes contained growth and in one of these was a follicle of histiocytes containing a few giant cells but no anisotropic material. Fibrosis was not a conspicuous feature of the follicles in the

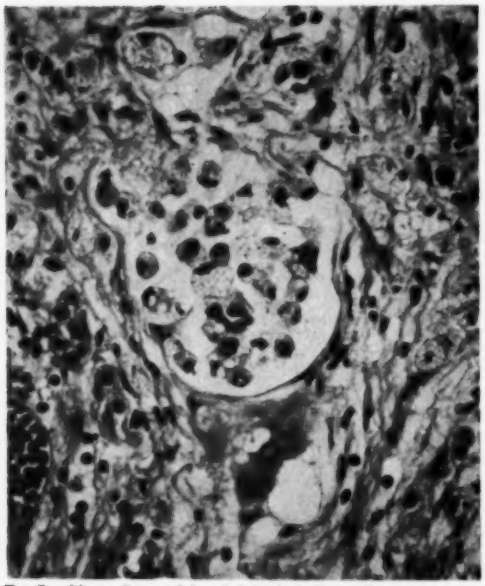

FIG. 7.—Giant cell, containing globular inclusions, adjacent to tumour cells (haematoxylin and eosin × 350).

stomach or the lymph node, though each histiocyte and giant cell was surrounded by a reticulin strand.

Case 8 (S54/1398).—F. D., a woman aged 28, had a carcinoma of the cervix with a giant cell reaction in an iliac lymph node.

This woman was admitted to hospital in March, 1954, for the investigation of a vaginal discharge. A carcinoma of the cervix was found on examination and treated by the insertion of radium. Post-radiation biopsies showed persistent viable growth and a Wertheim's hysterectomy was done in April, 1954. The iliac lymph nodes which were removed did not appear to contain growth macroscopically.

The patient left the country and has not been seen subsequently.

Histology.—The cervical tumour was a poorly differentiated epidermoid carcinoma. None of the iliac lymph nodes sectioned contained growth, but one of them showed a cluster of histiocytes and a few giant cells, without inclusions, near the periphery. There was no fibrosis in the node.

Case 9 (S54/1439).—K. S., a woman aged 51, had a carcinoma of the parotid gland with a giant cell reaction in a regional lymph node.

This woman was admitted to hospital in April, 1954, complaining of a lump in the region of the left parotid gland. Biopsy showed it to be a carcinoma. A course of *x*-radiation was given followed by removal of the entire parotid gland.

She was readmitted in June, 1955, because of recurrence of swelling at the operation site; this was removed and found, on histological examination, to be a lymph node. She subsequently developed widespread metastases and died at home. A necropsy was not obtained.

Histology.—The neoplasm in the parotid was a poorly differentiated carcinoma. The lymph node, removed in June, 1955, was infiltrated by sheets of polygonal carcinoma cells separated by strands of hyaline fibrous tissue; many vessels showed intimal fibrous thickening. Occasional ill-defined follicles of histiocytes were seen, containing giant cells, some of which had anisotropic material in the cytoplasm. Reticulin strands extended around the histiocytes and giant cells.

Case 10 (P56/478).—J. S., a man aged 64, had a carcinoma of the pinna with a giant cell reaction in the regional lymph nodes.

This man was first seen in 1953 with a carcinoma of the left pinna which had been present for five years. He refused operation and was treated by radiotherapy. The lesion recurred and he agreed to removal of the left pinna in May, 1954. The lesion recurred on two further occasions in November, 1954, and September, 1955; the growth was excised on both occasions. A block dissection of enlarged cervical lymph nodes was done in July, 1956. Recurrence of growth and infec-

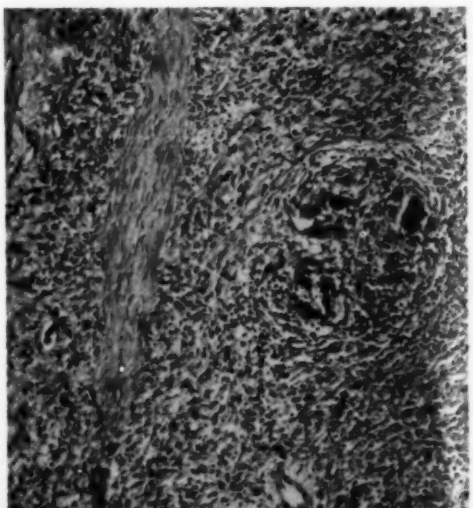

FIG. 8.—Circumscribed lesion in stomach wall (haematoxylin and eosin × 90).

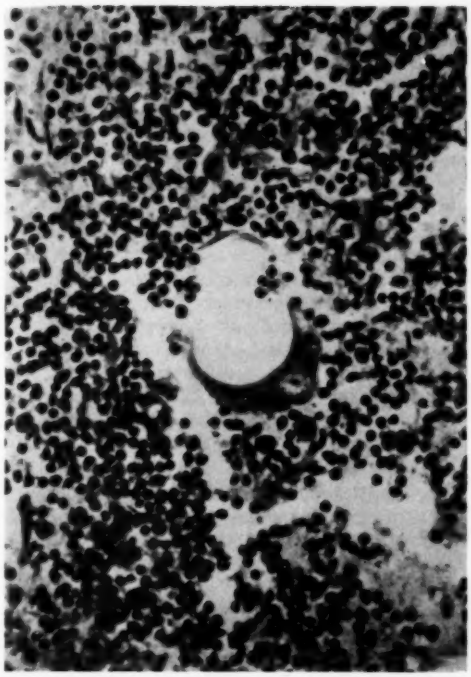

FIG. 9.—Giant cell with asteroid inclusion (haematoxylin and eosin × 350).

tion occurred at the site of excision of the primary and secondary neoplasms; he developed bronchopneumonia and died in December, 1956.

Necropsy showed masses of firm white growth around the left external auditory meatus and extending downwards to surround the carotid arteries and medially involving the transverse processes of three upper cervical vertebrae. Growth was seen macroscopically in left and right cervical and mediastinal lymph nodes. Small metastases were present in the lower lobes of both lungs, which also showed areas of bronchopneumonia. No metastases were found elsewhere.

Histology.—Ten lymph nodes removed in June, 1956, all contained numerous rounded collections of histiocytes and giant cells; inclusions were not seen in the latter. A few strands of fibrous tissue were present around the follicles, but reticulin fibres did not extend between the histiocytes in the follicles. An occasional follicle showed a slight degree of central necrosis and haemosiderin was present, situated near to the follicles in many of the nodes. Numerous lymph nodes were taken at necropsy from the neck, thorax, axillae, and abdomen. Some contained growth, but none contained giant cell lesions.

Case 11 (P55/216).—J. M., a man aged 59, had a giant cell reaction in an omental lymph node which was an incidental finding at necropsy.

This man suddenly collapsed and died. Necropsy revealed a myocardial infarct due to coronary thrombosis. A slightly enlarged lymph node was noted in the lesser omentum; other nodes and the spleen were normal.

Histology. — Numerous giant cells containing asteroids and globular bodies were seen in the sinuses throughout the node (Fig. 9). There was no histiocytic proliferation nor was there any fibrosis.

Discussion

Granulomatous lesions, histologically similar to those seen in sarcoidosis, have been reported in association with carcinoma (Wolbach, 1911; Gherardi, 1950; Nadel and Ackerman, 1950; Symmers, 1951; ten Seldam, 1956). Cowdell (1954) discusses such lesions in the differential diagnosis of sarcoidosis. Collections of epithelioid and giant cells are frequently found in the ovarian disgerminoma (Schiller, 1934; Föderl, 1938; Sailer, 1940), and occasionally in granulosa cell tumours (Schattenberg and Harris, 1946). Sailer (1940) has reviewed the theories of origin of such lesions, which are tuberculosis, a stromal reaction to disintegrating tumour cells, or that they may be derived from the neoplastic cells.

The association of sarcoidosis with malignant neoplasm is rare; Case 1 could be an example of this. There are, however, certain features of this case which are not in agreement with a diagnosis of sarcoidosis. There are histological differences between the lesions in the gastric nodes, which resemble those of sarcoidosis, and those in the lung, liver, and other lymph nodes which conform to the criteria of Stengel-Wolbach sclerosis. The spleen was not involved; this makes a diagnosis of Stengel-Wolbach sclerosis or sarcoidosis improbable. Case 1, therefore, could either be a widespread granulomatous reaction to carcinoma of the stomach and its products, or else the coexistence of a gastric neoplasm and sarcoidosis. It seems unlikely that Stengel-Wolbach sclerosis can be considered to be histologically distinct from sarcoidosis. In Case 1, lesions which resembled sarcoid and Stengel-Wolbach sclerosis were found. The lesions in regional nodes from Case 5 were also of Stengel-Wolbach type, but no other evidence of this disease was present.

The remarkable localization of lesions, resembling Stengel-Wolbach sclerosis, to the spleen and para-aortic lymph node in Case 2 makes a diagnosis of Stengel-Wolbach sclerosis improbable.

It seems more likely that those in the spleen were due to related clumps of haemosiderin or anisotropic material which was also present in some giant cells in the lymph node; Refvem (1954) reports sarcoid-like lesions produced by injection of blood. The lesions in Cases 5 and 10 may also be due to haemosiderin. Marshall (1956) has suggested that lesions seen in sarcoidosis and Stengel-Wolbach sclerosis are variants of the same disease process. Furthermore, the findings in these cases suggest that both types of lesions may also occur as a tissue response to such agents as blood (Cases 2, 5, and 10), foreign material (Cases 2, 7, and 9), and products of necrosis of neoplasms (Cases 1, 3, 4, and 8).

No obvious cause was found for the lesions in the involved lymph node taken from the abdomen of Case 11. Four other cases in this paper showed lesions in abdominal nodes, and, though this may be coincidental, the possibility remains that foreign material or blood may make its way through minor breaches in the mucosa of the gut and cause such lesions in lymph nodes. The finding of sarcoid-like lesions, related to foreign material, in regional lymph nodes from Crohn's disease lends support to this view. However, a survey of the regional nodes from 50 cases of gastric ulcer examined in this laboratory over the past year failed to show any such lesions; this may be partly due to the smaller number of lymph nodes sectioned as compared to those examined in gastric carcinoma and partly to the restriction of this type of tissue reaction to certain individuals.

In Cases 3, 4, and 9 lesions were seen in the peripheral sinus of the nodes: this suggests that the exciting factor may be transported by the lymph stream. Symmers (1951) supports this view, though he reports the frequent finding of lesions near the hilum of the nodes. In Cases 3 and 8 the regional nodes and the neoplasm itself had been treated with *x* rays; this, no doubt, contributed to the amount of necrotic material reaching the nodes.

Asteroid or globular inclusions, in giant cells, cannot be considered to be diagnostic of either Stengel-Wolbach sclerosis or sarcoidosis. Goldmann (1890) illustrates an asteroid body in a giant cell found in a retro-auricular dermoid cyst, and Wolbach (1911) found such inclusions in giant cells in lymph nodes related to carcinoma. The origin of asteroids from globular inclusions was suggested by Friedman (1944), who found both to have similar histochemical properties. Teilum (1949) made the observation that Schaumann bodies were also closely allied histochemically to asteroid inclusions and postulated the asteroid as the precursor of the double contoured bodies of Schaumann.

The presence of asteroid and globular inclusions in the nodes from Cases 1, 2, 5, and 11, and of globular inclusions alone in Cases 3 and 6, suggests that they are non-specific structures which cannot be considered as unique features of any one disease process.

The cases reported in this paper illustrate a variety of granulomatous reactions in lymph nodes related to, or distant from, malignant neoplasms. The histological appearances resemble sarcoidosis in some, Stengel-Wolbach sclerosis in others; in the remainder the lesions are not reminiscent of either. This diversity may in part be due to the neoplasm; in this series gastric carcinoma is a factor in four cases. Another factor may be host idiosyncrasy.

The findings emphasize the fact that sarcoidosis is a disease entity, not a histological one, a point which must be borne in mind when assessing the value of conjunctival, scalene node biopsy (ten Seldam, 1956) and other forms of biopsy as an aid to its diagnosis.

Summary

Eleven cases are presented in which lymph nodes and sometimes other organs show histological features resembling sarcoidosis or Stengel-Wolbach sclerosis.

In all but one case a malignant neoplasm is present.

The histological features are discussed.

We are grateful to Dr. A. M. Barrett for his encouragement and advice and for permission to utilize the histological material. The consultant staff of Addenbrooke's Hospital kindly allowed access to the case histories. Mr. S. W. Patman prepared the photographs.

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TRANSPLACENTAL BLEEDING FROM THE FOETUS*

BY

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The suggestion that the foetus can become severely anaemic from haemorrhage into the maternal circulation was first made by Wiener (1948). A case reported by Wickster (1952) was almost certainly due to such a haemorrhage. However, neither Wiener nor Wickster made confirmatory laboratory tests, and it was Chown (1954, 1955) who first proved, both serologically and by showing excess of alkali-resistant haemoglobin, the presence of foetal cells in the blood of a woman just delivered of an anaemic child. A case in which anaemia was slight, but serological evidence strong, was reported by Dunsford (1957). The presence in the maternal blood of an excess of alkali-resistant haemoglobin without serological tests has been interpreted as an indication of transplacental bleeding by Colebatch, Pitt, and Maddison (1956) and by O'Connor, Shields, Kohl, and Sussman (1957).

Two cases are described here of neonatal anaemia in which foetal cells were demonstrated in the maternal blood by differential agglutination and other tests. The first infant had severe haemolytic disease; the second severe anaemia which appeared to be due entirely to transplacental bleeding.

Case Reports

Case 1.—Mrs. C. M., aged 30, was delivered at 4.50 p.m. on February 28, 1955, in the 37th week of her pregnancy, of a pale, shocked, hydropic female infant. Shortly thereafter she had a rigor. Her first child, born in 1947, was normal; the second (1952) died at the age of 2 days from haemolytic disease; the third (1953) was stillborn and hydropic. She had felt well after delivery of the first child, but with the second and third, as with the present fourth, she had a rigor after delivery. The present pregnancy had been normal apart from the continued presence in the serum of rhesus antibodies (anti-D). At the 36th week the anti-D titre was 1 in 8 in saline and 1 in 256 in albumin. She was group B, Rh negative (C−, D−, E−, c+). The labour was spontaneous and there was no significant external bleeding (total loss 4 oz.). The cord was ligated immediately after delivery of the child. The placenta was large and oedematous with ragged membranes.

Laboratory Investigations.—The cord blood was group B, Rh positive (D positive). A direct Coombs test was positive. Haemoglobin was 24% (3.6 g.), R.B.C.s 1.18 m., P.C.V. 15%, M.C.V. 127 cu.μ, M.C.H.C. 24%, reticulocytes 23%. Plasma showed severe icterus.

Blood films showed severe erythroblastaemia (70% of all nucleated cells) and much polychromasia, and myelocytes and a few monocytic erythrophages among the white cells. Platelets were scanty. The diagnosis was severe haemolytic disease.

The mother's blood on the day after delivery showed Hb 85% (12.6 g. per 100 ml.), P.C.V. 38%, and M.C.H.C. 33%. A film was within normal limits.

The fact of the rigor after delivery suggested that blood from the foetus had leaked into the maternal circulation and caused a minor transfusion reaction (Goodall, 1955, 1957). Therefore the buffy coat was examined for evidence of ingested red cells, films being made from a freshly spun sample and from one incubated at 37° C. for one hour. Erythrophagocytosis was found in both, especially in the latter where there were 87 erythrophages per 10,000 potential phagocytes, an erythrophagocytic ratio of 0.87% (normal up to 0.10%, Zinkham and Diamond, 1952). Most ingested red cells were in neutrophil polymorphs (see Fig. 1), the rest in monocytes. One ingested normoblast was seen.

Erythrophagocytosis is known to occur in haemolytic anaemias (Zinkham and Diamond, 1952) and in megaloblastic anaemia of pregnancy (Goodall, 1957), but this mother was not anaemic. Thus, in view of the possibility that the ingested cells might be foetal, the following tests were made in the maternal blood.

A direct Coombs test showed, in a general background of unagglutinated cells, clumps (6–8 cells) that were accepted as sensitized cells.

* Shortened versions of this paper were given to the Caledonian Branch of the Association of Clinical Pathologists in November, 1956, and to the Pathological Society in January, 1957.

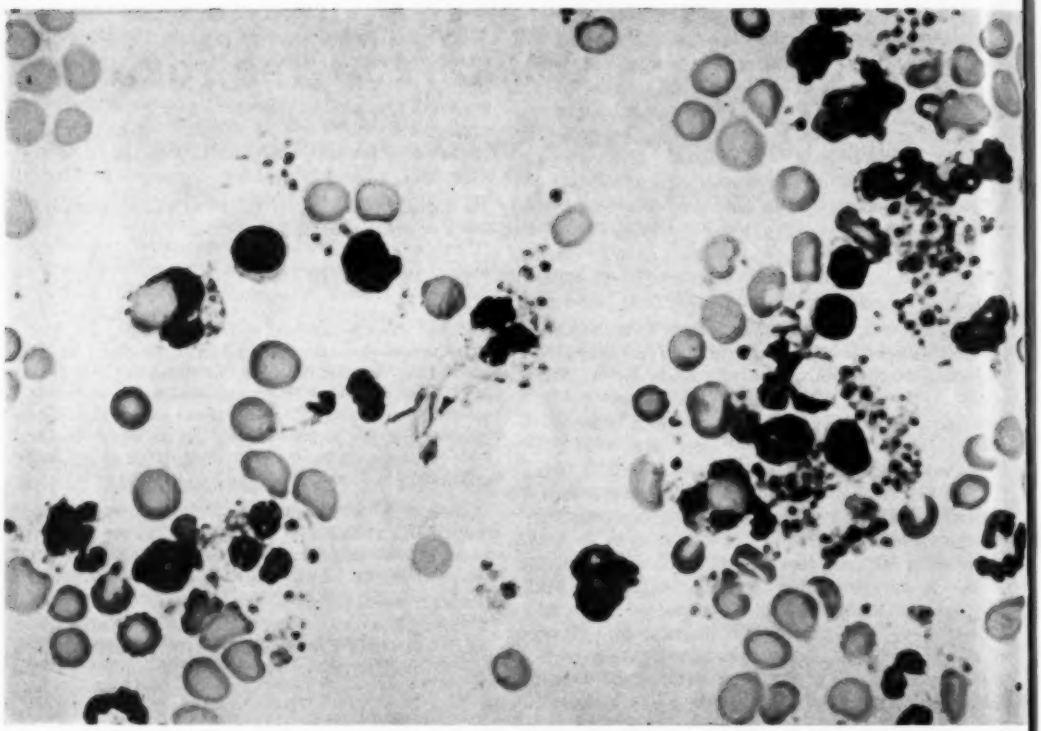

FIG. 1.—This smear from the maternal buffy coat in Case 1 shows two neutrophil polymorphs with ingested red cells; the large vacuole in another cell is possibly the end-result of similar phagocytosis. Leishman, × 800.

On incubation with strong "saline" anti-D, small scattered clumps were present with two different anti-D sera after two hours' incubation at 37° C.

Alkali-resistant haemoglobin was estimated by the method of Singer, Chernoff, and Singer (1951). There was no increase of alkali resistance as compared with normal controls.

The first two tests indicate that sensitized Rh-positive cells were present in the blood of the Rh-negative mother. It is difficult to assess the number of these presumed foetal cells. The clumps were about the size (6 to 8 cells) which Mollison (1956) observed with agglutinable cells in a concentration of 1 in 200. Accepting Mollison's figure, it can be said (on six agglutination counts in a Neubauer chamber) that the proportion of foetal cells in the maternal blood was probably no higher than about 1 in 200. This is supported by the virtual absence of foetal haemoglobin (alkali resistance). It should be remembered, however, that this proportion was found on the day after the delivery and rigor, by which time many of the incompatible cells could have been destroyed. As for indirect methods of assessing the magnitude of the "transfusion reaction," the plasma on the day after delivery was neither jaundiced nor tinged with haemoglobin and the urine did not contain abnormal pigments.

Progress of the Mother.—She remained well throughout the puerperium, with no fever or sign of infection, toxaemia, or jaundice. The urine was normal. On the third, fifth, and ninth days after delivery blood samples showed no significant erythrophagocytosis, negative differential agglutination tests, and virtual absence of alkali-resistant haemoglobin.

Progress of the Child.—Despite exchange transfusion jaundice increased and the child died the day after birth.

Necropsy (Dr. K. Rhaney).—This showed severe haemolytic disease with haemorrhagic manifestations, but no evidence of kernicterus. The plasma bilirubin level was 10.7 mg. per 100 ml.

Histology.—Findings were in keeping with the clinical and anatomical diagnosis. Siderosis of the spleen was largely concentrated in the capsule and trabeculae.

The appearance of the placenta was compatible with haemolytic disease. A few small groups of normoblasts (presumably foetal) were found in intervillous spaces.

Case 2.—Mrs. N. M., aged 24, gave birth to her second child, a mature female, on April 19, 1956. The first pregnancy and delivery were normal and the child was healthy. She was well during the second pregnancy, apart from an easily corrected retroversion of the uterus at 10 weeks' gestation. Labour was normal with a blood loss of 8 oz. The cord was tied after pulsation had stopped with the child slightly lower than the uterus. The infant was pale and shocked with peculiar sighing, rapid respirations suggestive of air hunger, yet no obvious haemorrhage was found. There was no evidence of jaundice, petechiae, ascites, splenomegaly, hepatomegaly, or skeletal deformity. The placenta showed no abnormality on routine examination, in particular, neither retroplacental clots nor vascular anomalies were seen. It was not kept for microscopy. The mother's blood group was O, Rh positive, and her Wassermann reaction negative.

Laboratory Investigations.—Preliminary investigation of the infant's blood (heel stab) on April 19 showed Hb 39% (5.8 g.), total nucleated cells 43,600 per c.mm. A differential count of 500 nucleated cells gave: neutrophil polymorphs, 63.8% (27,817 per c.mm.), eosinophil polymorphs 0.0%, basophil polymorphs 0.0%, neutrophil metamyelocytes 3.2% (1,395), neutrophil myelocytes 1.4% (610), monocytes 4% (1,740), lymphocytes 18.6% (8,110), normoblasts 7.8% (3,400), smears 1.2% (523).

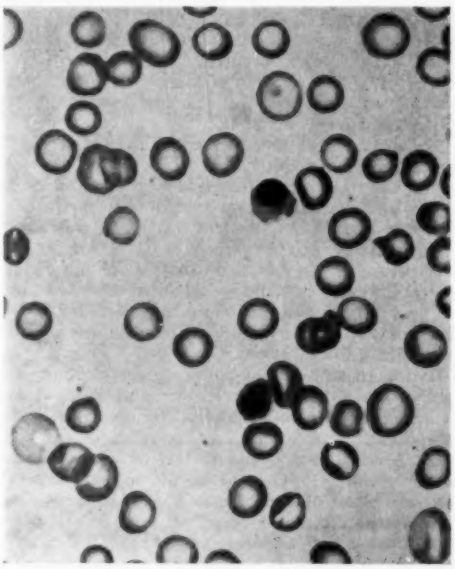

FIG. 2.—This film of the infant's blood in Case 2, taken shortly after birth, shows numerous small erythrocytes with excessive central pallor. Two tiny normoblasts are seen. Leishman, × 800.

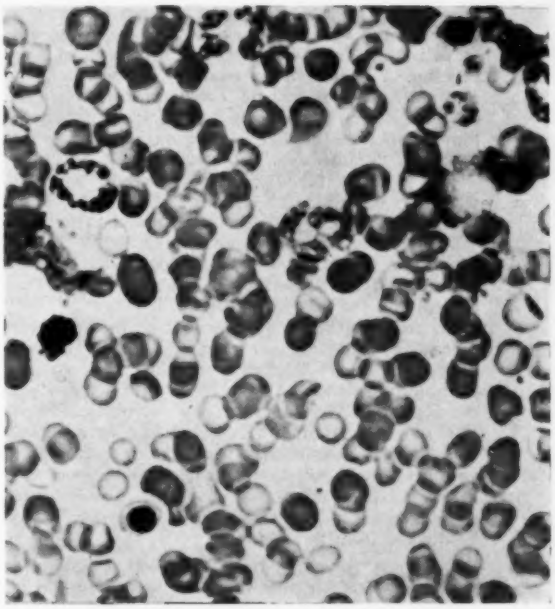

FIG. 3.—The maternal buffy coat in Case 2 contains many small, ring-stained erythrocytes similar to those seen in the infant's blood (Fig. 2). Tiny normoblasts are also present here. Leishman, × 800.

On a film the red cells were generally smaller and more hypochromic than normal, and the normoblasts small with pyknotic nuclei and scanty, hypochromic cytoplasm (Fig. 2). Polychromasia was more obvious than in a normal infant. There was a neutrophil polymorphonuclear leucocytosis. Platelets appeared normal. A direct Coombs test was negative. Blood was group O, Rh positive. The plasma bilirubin level was 2.1 mg. per 100 ml.

Haemolytic disease due to rhesus incompatibility could be excluded by the negative direct Coombs test, and there was no ABO incompatibility; thus a haemolytic basis was unlikely. There was nothing to suggest any primary disease of the haemopoietic system. The presence of tiny, hypochromic, pyknotic normoblasts in a generally microcytic, hypochromic red cell population recalled a chronic post-haemorrhagic anaemia, while the very low haemoglobin level just after birth also suggested that bleeding had taken place some time before delivery. Nevertheless the clinical evidence of acute post-haemorrhagic shock and the neutrophil leucocytosis were in keeping with more rapid, recent haemorrhage. In sum, the evidence

pointed to a post-haemorrhagic anaemia of considerable duration with a final exsanguination at the time of delivery. Thus, since there was no sign of local bleeding or haemorrhagic tendency in the infant, no retroplacental clot and no antepartum, intrapartum, or postpartum haemorrhage from the mother, we thought that the mother's blood might be shown to contain a "transfusion" of foetal cells.

The mother's blood was examined on April 20, when Hb was 83% (12.3 g.), P.C.V. 37%, M.C.H.C. 33%, and a film of the red cells showed them to be generally normochromic, normocytic, with a small proportion of microcytes, some hypochromic, and an occasional pyknotic normoblast. The leucocytes were normal and the platelets adequate.

On buffy coat films, hypochromic normoblasts were present in large number (Fig. 3), but no erythrophagocytosis. The presence of tiny, hypochromic, pyknotic normoblasts in the blood of a woman who did not herself have hypochromic microcytic anaemia was certainly unusual. Its occurrence in a woman who had just given birth to a child with severe and as yet unexplained post-haemorrhagic anaemia and with the same type of normoblasts in its blood gave further support to the idea that these nucleated red cells must be derived from the foetus.

Serological Investigation.—Both mother and infant were group O, Rh positive (D+), and there was no evidence of iso-immunization. Nevertheless more extensive rhesus typing was undertaken in the hope of finding some difference in group that would allow differential agglutination tests on the mother's blood. Table I shows the results.

TABLE I
BLOOD GROUP DATA IN CASE 2

ABO Group	Rhesus Group				
	(Anti) C	(Anti) D	(Anti) E	(Anti) c	Probable Genotype
Baby M, O	+	+	−	+	CDe. cde (R_1r)
Mr. M, O	−	+	+	+	cDE. cde (R_2r)
Mrs. M, O	+	+	−	±*	CDe. CDe (R_1R_1)

* On August 10, 1956, and subsequently Mrs. M. was completely negative with anti-c.

The result with our anti-c serum on the mother's blood is the important one. This particular serum was known to be very avid when used against red cells suspended in albumin and in the indirect Coombs test. With both techniques the mother's blood showed scattered clumps in a background of unagglutinated cells. A sample field from the anti-c albumin test is shown in Fig. 4. The indirect Coombs test showed slightly larger agglutinates. Fig. 5 shows a simultaneous control using known CDe/CDe cells showing complete absence of agglutination. The infant's blood obtained before transfusion was similarly tested, and, as shown in Fig. 6, was almost completely agglutinated, incidentally showing that it contained no appreciable number of maternal cells. Thus, though the maternal blood was generally c-negative, it contained c-positive cells. As the foetal cells were

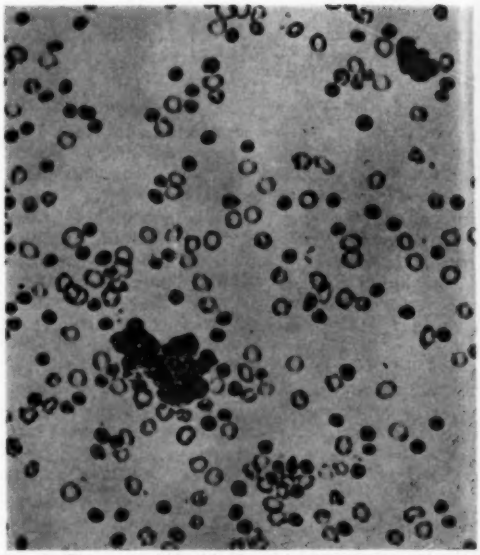

FIG. 4.—The maternal blood in Case 2 has been treated with anti-c (albumin method) and shows the small agglutinates which are presumably foetal cells. × 340.

known to be c-positive, and the mother was not a twin and had never been transfused, it was considered that this partial agglutination of the mother's blood with anti-c was a confirmation of the presence of foetal cells in her circulation. It was shown on August 10, 1956, and subsequently that the mother was indeed c-negative and that the foetal cells had disappeared from her blood.

An attempt was made to assess the proportion of c-positive cells in the blood of the mother by comparing the results of differential agglutination on her blood and on prepared proportionate mixtures of CDe/cde and CDe/CDe cells. While it was almost certain that at least 5% of c-positive cells must have been present in the mother's blood, it was not possible to give an exact estimate by this method.

There was not at this time any evidence of iso-immunization in the mother's serum (saline, albumin, and indirect Coombs test).

Results in other blood group systems are not given here as the groups of the mother and child were so similar that they were useless for practical differentiation.

On April 20 a preliminary test by the method of Singer *et al.* (1951) showed the presence in the mother's blood of approximately 5% alkali resistance in excess of the maximum alkali resistance found in 10 normal blood donors. On May 4 the mean of four such estimations showed 5.9% excess of alkali resistance above the maximum found in 30 normal blood donors and 15 normal pregnant women. These findings gave further evidence of foetal cells in the mother's blood.

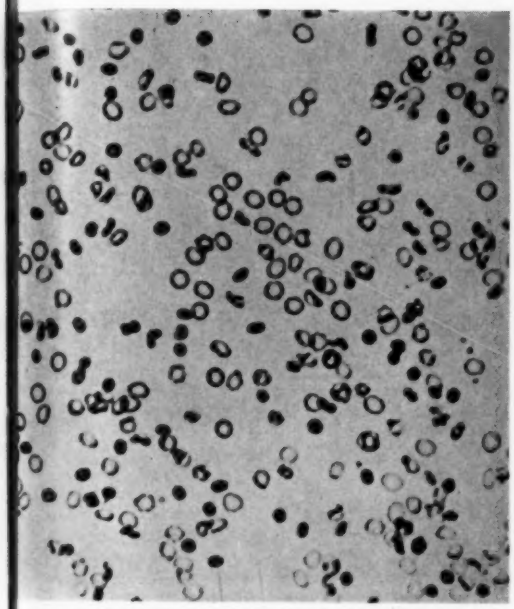

FIG. 5.—This is the control for Fig. 4, these erythrocytes being c-negative (CDe/CDe; R_1R_1). The mother was later shown to be of this very group. × 340.

Progress of Child.—An initial small transfusion relieved shock, but did not materially affect the blood picture. A venous sample showed Hb 37% (5.5 g.), reticulocytes 5.8%. In order to raise the haemoglobin without causing too great an increase in blood volume an exchange transfusion was carried out on April 20 (Dr. J. Doyle). The amount withdrawn was 450 ml., the amount transfused 360 ml., the deficit being necessitated by overloading of the right heart, as shown by tachycardia and venous engorgement. On April 22 the Hb (heel stab) was 65% (9.6 g.). The Hb level remained almost the same for two months, but rose thereafter (see Table II). No iron was given to the child, which was breast-fed, with later supplements, but the mother took iron (ferrous gluconate, gr. 5, once daily). The child gained weight normally, and, when last seen on July 2, 1957, was in good health.

TABLE II

PROGRESS OF CASE 2 MEASURED BY HAEMOGLOBIN LEVELS

Date	Haemoglobin (g. per 100 ml.)
19/4/56 (small transfusion)	5·8
20/4/56 (exchange transfusion)	5·5*
22/4/56	9·6
26/4/56	10·1
15/6/56	9·4
22/6/56	9·6
13/7/56	12·0
10/8/56	12·9

* Venous sample, all others heel stab.

Follow-up of Changes in Maternal Blood.—The detection of transfused cells by differential agglutination is only of limited sensitivity. Thus Mollison (1956) states that this reaction is of use only if the concentration of readily agglutinable cells is of the order of at least 1 in 200. Wiener (1942) is not so optimistic. The alkali resistance test is an even less sensitive index of the presence of foetal cells, as up to nearly 2% of alkali-resistant haemoglobin may be present in normal blood (Singer *et al.*, 1951). It was thought that spinning the mother's blood might concentrate the foetal erythrocytes in a particular layer of the packed cells. This was done in Wintrobe tubes at 3,000 r.p.m. for 30 minutes and the packed cells divided into eight approximately equal portions. The fractions were tested with anti-c in albumin and by the indirect Coombs test after incubation with anti-c. The results are shown in Table III and Figs. 7, 8, and

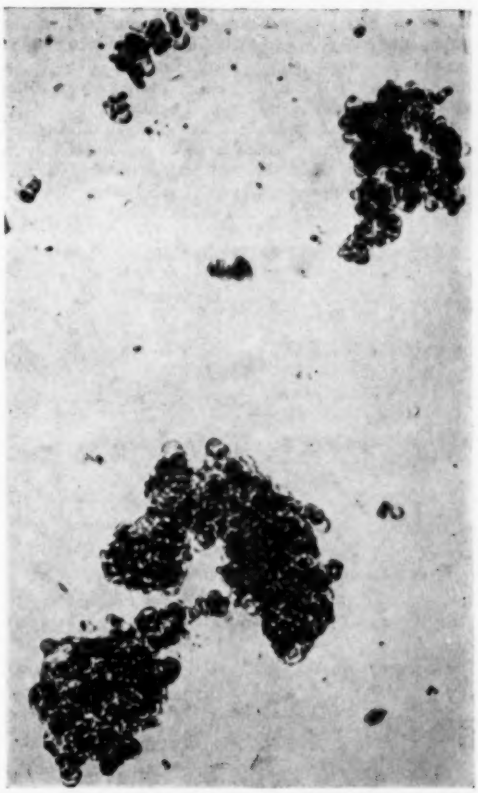

FIG. 6.—The infant's blood in Case 2 obtained before transfusion gave practically complete agglutination with anti-c. × 340.

TABLE III
RATE OF DISAPPEARANCE OF FOETAL CELLS FROM MATERNAL CIRCULATION IN CASE 2

Date	Method	Ordinary Sample	Centrifuged Fractions (from Above Down)			
			1 to 5	6	7	8
4/5/56	D.A.	+	0	(+)	+	++++
	A.R.	5·9	Not done			
22/6/56	D.A.	(+)	0	(+)	+	++
	A.R.	0	0	0	0·5	4·3
13/7/56	D.A.	? trace	0	0	(+)	(+)
	A.R.	0	0	0	0	0
10/8/56	D.A.	0	0	0	0	0
	A.R.	0	0	0	0	0

D.A. = Differential agglutination tests using anti-c.
A.R. = Alkali-resistant haemoglobin in excess of normal maximum expressed as a percentage of total haemoglobin (see text).

9. The symbol + is used to denote the degree of agglutination in the general sample tested on May 4. The symbol (+) means definite agglutination, but of the order of not more than half of the original general sample. The results with the Coombs test were substantially the same as with the albumin suspension. As the fractions of packed cells were small, it was not possible to estimate their alkali resistance by the method of Singer *et al.* The technique is shown in the Appendix. The mean results are given in Table III with the results of differential agglutination.

From these data it is clear that differential agglutination was a more sensitive index of the presence of foetal cells than was the estimation of alkali-resistant haemoglobin, though it is obvious that the two tests run in parallel and thus confirm each other.

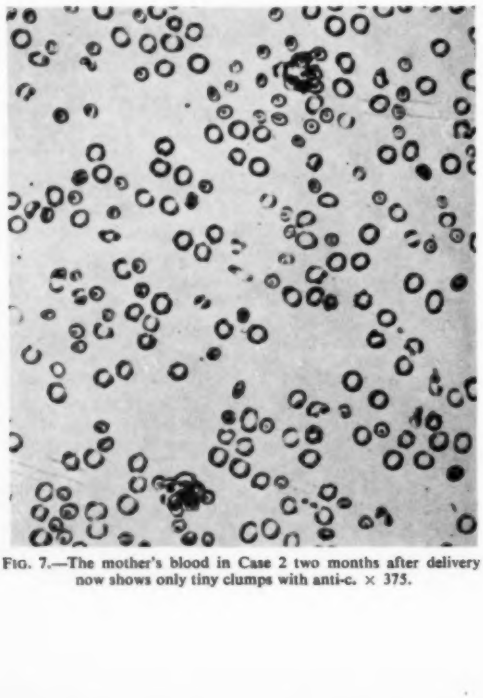

FIG. 7.—The mother's blood in Case 2 two months after delivery now shows only tiny clumps with anti-c. × 375.

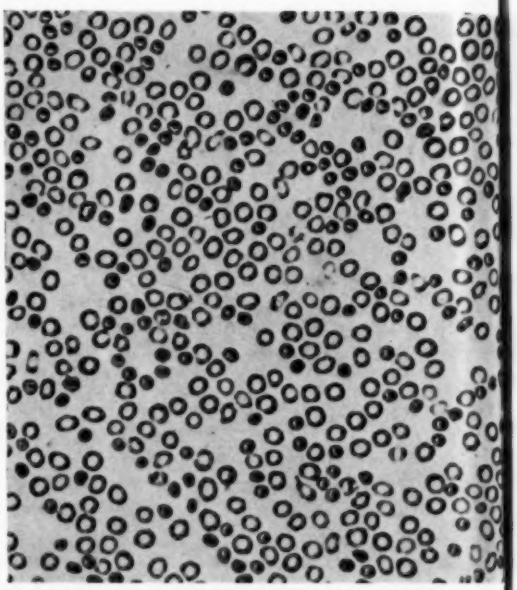

FIG. 8.—The result of centrifugation on the previous sample (Fig. 7) shows in the top layer no clumps with anti-c (cf. Fig. 9). × 375.

It also appeared that the foetal cells at this later stage in their ectopic intramaternal life were concentrated in the lower part of the haematocrit. On July 13 the results of agglutination with anti-c, recorded as "? trace," would undoubtedly have been recorded as negative had one not looked very intently over several samples from the tube. Thus, 12 weeks after delivery, the ordinary sample was for all practical purposes negative whereas the two lowest samples from the haematocrit were definitely positive. It must be remembered, however, that, one day after delivery, there were tiny normoblasts, presumably foetal, in the buffy coat at the top of the packed cells. It is considered that the foetal cells became more dense as they matured and thus were concentrated in the bottom of the haematocrit.

Throughout the investigation serum from each sample of maternal blood was tested by the indirect Coombs method for the presence of anti-c, but results have been entirely negative.

Subsequent Pregnancy.—The mother was delivered of another daughter on July 3, 1957. The pregnancy was uneventful and the maternal blood showed no evidence of foetal cells or iso-immunization before or after delivery. The infant is not anaemic.

To summarize, an infant was born with severe anaemia which could be explained only by massive haemorrhage from the placental vasculature of the foetus into the maternal circulation. This was confirmed by the presence in the maternal blood of

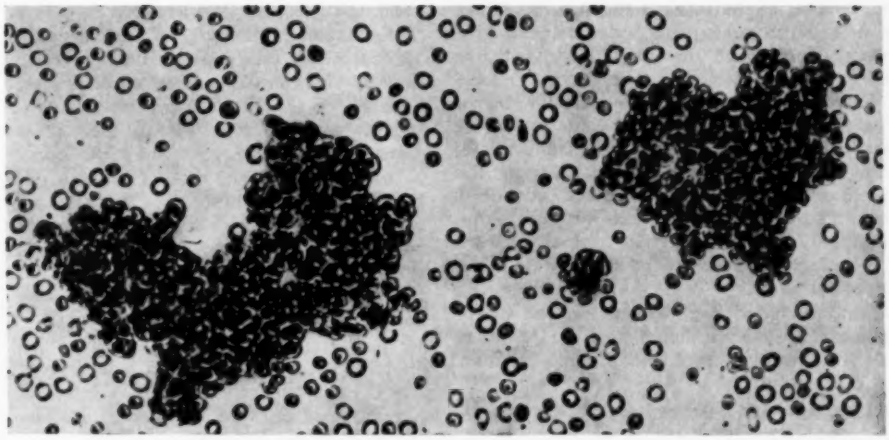

FIG. 9.—The lowermost layer with anti-c (same time as Figs. 7 and 8) shows more agglutination than the ordinary sample did at the time of delivery, two months previously (see Fig. 4). × 375.

nucleated red cells not associated with maternal anaemia and similar in type to those in the foetus, by differential agglutination and by the finding of a significant amount of alkali-resistant haemoglobin in the mother. The nature of the placental lesion allowing this leakage of blood was not ascertained as the placenta was not available for detailed study.

The proportion of foetal red cells in the maternal blood just after delivery was more than 5%, possibly nearly 10%. From weight and height the mother's blood volume was estimated as approximately 4.5 to 5 litres, while that of the infant, which weighed 6 lb. 4 oz. at birth, should normally have been about 250 ml. Thus the amount of blood lost from the foetus must have been at least that of its expected blood volume. Such a large amount of blood must have been lost over a considerable period of time, otherwise the foetus would have died *in utero*. Confirmation of the long duration of the bleeding arises from the facts that the child's haemoglobin was very low at birth and that the hypochromic microcytic erythrocytes and tiny erythroblasts resembled exactly those of chronic blood loss at other periods of life.

Discussion

Until the discovery of the relationship between the rhesus blood groups and haemolytic disease of the newborn, little was understood of neonatal anaemia, and the prognosis was poor. As knowledge of haemolytic disease has increased, it has also become apparent that haemorrhage from the foetus, though less common than haemolysis, is nevertheless an important cause of anaemia. Any systematic classification of the causes of such bleeding tends to be rather lengthy, as the causes are numerous (Wickster and Christian, 1954), but it is convenient for the clinician to group them either as overt, if haemorrhage was observed at delivery, or occult, as in the two cases reported here. The site of bleeding may be the placenta, the umbilical vessels or anomalous vascular connexions, or the foetus itself.

Clinical Diagnosis of Post-haemorrhagic Anaemia of the Newborn.—The main clinical features in the infant, as summarized by Colebatch *et al.* (1956), are pallor, weakness, loss of muscle tone, tachycardia, absence of jaundice, absence of enlargement of liver and spleen, failure to revive with oxygen, but a dramatic response to transfusion. Given such signs and an obvious source of haemorrhage, the diagnosis is easy. When the bleeding is occult only particular regard to the character and rate of respiration will prevent the misdiagnosis of asphyxia pallida or intracranial injury.

Examination of the Placenta.—A thorough inspection of the placenta, cord, and membranes is essential, for, even when haemorrhage has not been obvious clinically, the naked-eye appearances may give an important clue, as in Chown's cases (1955).

Normoblasts in groups were found within the maternal intervillous lake by Kline (1948) and by Javert and Reiss (1952). These were presumed to be foetal cells, and to occur in clumps only if the number of erythroblasts in the foetus had been high, as in prematurity or haemolytic disease (Javert and Reiss).

Examination of the Infant's Blood.—A detailed study of the morphology of the cord blood or peripheral blood from the infant may well give important timous information, as in the second case, where the presence of a hypochromic microcytic blood picture was an indication not just of haemorrhage but of prolonged haemorrhage, probably of several weeks' duration. In the second case the normoblasts numbered only 7.8% of the total nucleated cell count whereas in the predominantly haemolytic first case they were 70% ; a differential count therefore appears to be of diagnostic value.

Appropriate blood group data and the direct Coombs test provide further distinguishing information, and estimation of the plasma bilirubin may also be useful.

After even severe haemorrhage, if it occurred only at the time of delivery, the cord blood haemoglobin might well be normal, but there would be subsequent haemodilution, leucocytosis, and a normoblastic reaction ; thus in a clinically doubtful case one should look for haemodilution by serial haemoglobin estimations.

Proof of Foeto-maternal Transfusion.—The occurrence of a rigor or a febrile reaction in the mother, as in Chown's Case 3 (1955) and the present Case 1, may well be a sign that incompatible foetal blood had entered her circulation, but this is of value only if infection can be completely excluded, and it must be remembered that amniotic embolism can cause a rigor. If a relatively large amount of incompatible foetal blood were to cross the placental barrier, haemoglobinuria, anuria, and jaundice might occur, but in the absence of incompatibility it is most unlikely that any evidence of foeto-maternal transfusion would be detected clinically in the mother.

Laboratory Investigation of Maternal Blood.—Examination of the buffy coat may show a significant degree of erythrophagocytosis (as in Case 1) or the presence of otherwise unexplained normoblasts (as in Case 2). Many cases of megaloblastic anaemia show numerous erythrophages in the buffy coat, but confusion is not likely to arise since examination of the buffy coat is a direct way to diagnosing the megaloblastic anaemias of pregnancy (Goodall, 1955, 1957). The many tiny normoblasts in the buffy coat of Case 2 pointed to foeto-maternal bleeding because the mother herself was not anaemic. These diagnostic cells were in fact seen in the ordinary blood films, where they were accompanied by more numerous, non-nucleated hypochromic microcytes.

The phagocytosis of foetal red cells in cases of rhesus incompatibility may well depend on an opsonin effect produced by the antibody, as for example the anti-D in the maternal serum of Case 1. It should be mentioned, however, that anti-Rh sera differ greatly in their property of promoting erythrophagocytosis of Rh-positive cells (Conway, 1953 ; Harkink, Doorman, and van Loghem, 1953 ; Bonnin and Schwartz, 1954) ; in fact many are inactive and others relatively weak as compared with anti-A or anti-B, especially lytic anti-A. Thus the occurrence of haemorrhage into a rhesus-negative mother with antibodies might not be followed by erythrophagocytosis. On the other hand, when an ABO incompatibility exists between foetus and mother (a heterospecific pregnancy) foeto-maternal haemorrhage would almost certainly lead to rapid ingestion of the foetal cells by the maternal leucocytes.

The most valuable serological test is differential agglutination, which, of course, depends on the presence of a different antigen or antigens in the maternal and foetal cells. The possibility of carrying this out depends on the availability of sera containing the relevant antibodies. The maternal blood is incubated with the appropriate serum in saline and in albumin, and the indirect Coombs test is performed. If the mother is immunized to the foetal cells (as in Case 1) the direct Coombs test on her blood may give valuable information by revealing the clumps of affected cells. Fractionation of the maternal blood by differential centrifuging may enable one to obtain a greater concentration of foetal cells in one or more fractions as in Case 2.

Changes in maternal antibody titre during pregnancy may give a useful indication that foeto-maternal bleeding has occurred ; postpartum changes certainly can, as in the case described by Dunsford (1957). Such changes can be taken only as presumptive evidence, but suggest that other tests are worth doing, as in Dunsford's case.

Adsorption of antibody *in vitro* is another possible indirect pointer, and Chown (1954) has described the adsorption of anti-D by an rh-negative mother's blood containing D-positive cells from the foetus.

The occurrence of anti-rhesus immunization after an episode of foeto-maternal bleeding is described by Chown (1954), but, though highly suggestive, this again is only presumptive evidence.

Of chemical tests, the most useful is the estimation of the alkali-resistant haemoglobin of the erythrocytes in the maternal blood. The widely

advocated "one minute" test of Singer *et al.* (1951) is a relatively simple method. This had to be modified for the fractionation experiments, because of the smallness of the available blood samples. None the less with adequate controls the modified though relatively crude method gave significant results. On the other hand, the qualitative type of investigation advocated by O'Connor *et al.* (1957) is not ideal. Their results appear to indicate that foeto-maternal haemorrhage is quite common, but a quantitative method seems to be required, preferably supported by other data. Thus a quantitative alkali-resistance test on the red cells in the maternal blood is an important part of the proof of transplacental bleeding, but care must be taken to obtain clear haemoglobin solutions (Singer *et al.*, 1951) free from red cell stroma (Iversen and Larsen, 1956). Caution must be exercised in the interpretation of the results of such estimations, and, if at all possible, this chemical method should be supported by morphological and serological evidence.

The estimation of alkali-resistant haemoglobin in the plasma of the mother is certainly worth doing if a mother has a rigor that might be due to the passage of incompatible cells from the foetus. This test has been done in a case of severe pre-eclampsia with haemoglobinaemia and haemoglobinuria: the haemoglobin in the plasma in this case showed no alkali resistance, thus excluding a foetal origin.

As for other tests for foetal haemoglobin, electrophoresis is known to be of no practical value in the detection of small proportions of foetal haemoglobin, and we have no experience of the immunological method used by Rucknagel and Chernoff (1955) or of the test recently described by Beaven, Ellis, and White (1956).

Conclusions.—Though the danger to the foetus of bleeding from itself, its cord, or its placenta has long been recognized, it is only since the discovery of the Rh factor and its relationship to haemolytic disease that this very different type of anaemia, post-haemorrhagic anaemia of the newborn, has received the attention it merits. Bleeding from the placental vessels of the foetus into the maternal circulation, postulated by Wiener and proved by Chown, is now confirmed and shown to be of possibly lethal extent. The confident confirmation of a suspected case by morphological, serological, and chemical methods is not beyond the resources of the routine clinical laboratory. Finally, it should be stressed that post-haemorrhagic anaemia of the newborn is an eminently treatable condition, and a condition which, when characterized by pallor and severe shock, requires transfusion without delay.

Summary

Two cases of transplacental bleeding from the foetus have been described; in the first the bleeding was slight and associated with severe haemolytic disease; in the second the bleeding was the cause of severe neonatal anaemia.

A description is given of the various methods of diagnosis suitable for use in a routine laboratory.

Post-haemorrhagic anaemia is an important cause of pallor in the newborn. Asphyxia pallida may be closely mimicked by post-haemorrhagic shock. Rapid diagnosis and treatment of the latter by prompt blood transfusion may save the life of an otherwise normal infant.

We are indebted to Professor Margaret Fairlie and the staff of the Maternity Department, Dundee Royal Infirmary, for their generous co-operation, and to Professors A. C. Lendrum and J. Walker for their help with this work. For clinical and other data we thank Professor J. L. Henderson, Dr. J. Thomson, Dr. K. Rhaney, and Dr. J. Doyle. Our thanks are also due to Miss Marjory Chalmers and Miss Aline Adam for technical assistance and to Mr. R. Smith for his help with the photography.

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APPENDIX

Method for Foetal Haemoglobin

The cells were washed three times in normal saline and lysed with distilled water. The solution was cleared by shaking with a third volume of toluene and centrifuging at 2,500 r.p.m. for 20 min. As this

did not always give a clear solution, filtration through a No. 1 Whatman paper was sometimes required, followed by further centrifuging. When only a small volume was available the solution had to be diluted with distilled water to get an adequate yield from filtration. These fractions and control haemoglobin solutions (30 normal blood donors, 15 normal pregnant women) were diluted with 0.007 N. NH_4OH until they gave a reading of 80 in the EEL photoelectric colorimeter using a 625 filter, and exactly 7 ml. quantities of the standardized solutions measured into tubes. The tubes were arranged at random, each being given only a serial number. Each solution had its reading of 80 checked. Then 0.5 ml. of N. NaOH was added and mixed for 30 sec. by a piston-shaped glass rod. With the top of the colorimeter closed the deflection was read at exactly one minute from the time of adding the NaOH. The alkaline solution was kept and read again in two hours, this reading being taken as the equivalent of complete conversion to alkaline haematin. Any excess of resistance between the one-minute reading of the fractions and the maximum one-minute reading among controls was considered as significant alkali resistance. By standardizing the EEL with the 625 filter and known concentrations of haemoglobin, it was thus possible to calculate the percentage of alkali resistance in excess of normal.

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SOME OBSERVATIONS ON THE COAGULATION DEFECT IN VITAMIN K DEFICIENCY

BY

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The coagulation defect produced by the coumarin drugs has been studied in recent years in great detail, but disturbance in the clotting mechanism produced by vitamin-K deficiency has been investigated to a much less extent. In everyday clinical practice a clotting disorder due to vitamin-K deficiency is now less common than that therapeutically induced by the use of coumarin or allied drugs.

It is generally accepted that the "hypoprothrombinaemia" produced by the coumarin drugs has a duel facet—a deficiency of prothrombin, the precursor of thrombin, and a deficiency of factor VII, a component required to react with tissue in the formation of extrinsic thromboplastin. It is also usually agreed that the serum of patients on the coumarin drugs has a defective reaction in the thromboplastin generating system, when interacted with normal adsorbed plasma and platelets.

It is the object of this paper to present evidence confirming that the vitamin K defect has the same features as those produced by the coumarin drugs and to show that at least one component of the blood thromboplastin defect is a deficiency of Christmas factor. This present study is complementary to recently reported observations (Douglas and Mair, 1957) of the Christmas factor defect in coumarin drug therapy.

Methods

Collection of Specimens.—Only specimens of blood obtained by clean venepuncture were used. The needles were of wide bore (s.w.g. No. 18). The syringes were allowed to fill without traction on the barrel and blood was collected therefore without frothing. For the preparation of plasma, venous blood was mixed with 3.8% sodium citrate in the proportion of nine parts of blood to one part of citrate, using graduated centrifuge tubes. For the preparation of serum, 10 ml. of venous blood was allowed to clot at room temperature in plain conical glass centrifuge tubes. The clotted blood was then stood in a water-bath at 37° C. for two hours and the serum separated by centrifugation.

Centrifugation.—This was carried out at 4° C. in a refrigerated centrifuge.

Glassware.—All the clotting times of fibrinogen or plasma were carried out in tubes of $\frac{1}{2}$ in. internal diameter maintained at 37° C.

Preparation of Reagents.—Human brain extract was prepared as described by Brown and Douglas (1952). M/40 and M/10 calcium chloride, 3.8% sodium citrate, aluminium hydroxide (alumina), and human fibrinogen were all prepared as described by Biggs and Macfarlane (1953).

Blood Examinations.—The whole blood coagulation time, the one-stage "prothrombin" time, recalcification time, and the tourniquet test were carried out by techniques described by Biggs and Macfarlane (1953).

The bleeding time was determined by Ivy's technique as described by O'Brien (1951). The platelet count was performed as described by Dacie (1956), and the factor V assay as described by Douglas and Biggs (1953) and Douglas (1956).

Thromboplastin Generation Test and Thromboplastin Dilution Curve.—These were done as described by Biggs and Douglas (1953) except that the serum was prepared as described above.

Two-stage Prothrombin Assay.—The globulin fraction technique as described by Douglas and Biggs (1953) was used.

Factor VII Assay.—This was determined as described by Douglas and Davies (1955) similarly to the method described by Biggs and Macfarlane (1953). The substrate plasma was obtained from patients on phenindione.

Prothrombin Consumption Technique.—Two procedures were used: (*a*) On whole blood as described by Douglas and Biggs (1953); (*b*) on recalcified plasma, being a comparison of the ability of normal or vitamin-K deficient plasma to correct the defective prothrombin consumption of recalcified Christmas disease plasma.

Citrated blood was collected from patients severely affected with Christmas disease. This blood was "low spun" at 500 r.p.m. for 20 min. and the platelet-rich plasma separated. The blood after removal of the "low-spun" plasma was centrifuged at 10,000 r.p.m. for five minutes on a high-speed head attachment. The resulting "high-spun" Christmas disease plasma was then added to the "low-spun" plasma. This manœuvre was designed to conserve the Christmas disease plasma.

"Low-spun" Christmas disease plasma, 0.5 ml., was delivered to each of 36 tubes. To 12 of these tubes (series A) was added a standard quantity of normal plasma, 0.05 ml.; a similar addition of vitamin-K-deficient plasma was added to a further 12 tubes (series B); a similar addition of Christmas disease plasma was added to the third set of 12 tubes (series C). For each of these three series of tubes (A, B, and C) there were 12 tubes containing 0.2 ml. saline, 0.2 ml. of brain, and 0.2 ml. M/40 calcium chloride and 24 tubes containing 0.2 ml. of fibrinogen. The tubes in each series (A, B, or C) were recalcified almost simultaneously with 0.125 ml. M/10 calcium chloride. At intervals of five minutes after recalcification one tube in each series was tested. Any fibrin formed was removed on a wooden applicator stick and 0.2 ml. added to the saline-brain-calcium tube. At this point a stop-watch was started, and at 45 seconds and 90 seconds thereafter 0.1 ml. was added to a 0.2 ml. amount of fibrinogen and the clotting time recorded.

Results

Two patients with vitamin K deficiency were studied.

The first was a 39-year-old woman with long-standing obstructive jaundice and a secondary steatorrhoea. Six years previously she had been operated upon for gall stones, the common bile duct ligated and divided, and a cholecystgastrostomy performed. Subsequently she developed jaundice and a further three operations were performed in attempts to provide drainage of bile into the upper intestinal track. These failed and she had in consequence obstructive jaundice and steatorrhoea. In consequence she developed typical deficiencies, night blindness, tetany, osteoporosis, and some haemorrhagic phenomena, menorrhagia, haematemesis, spontaneous bruising.

The whole blood coagulation time, bleeding time, tourniquet test, recalcification time, platelet count, and the factor V assay all gave normal results.

The one-stage "prothrombin" time was 27 sec. in the patient and 15 sec. in a control (Table I).

Thromboplastin Generation Technique.—The adsorbed patient's plasma was normal in the system. The serum gave a defective reaction (see Table II). In this table also is shown the failure of vitamin-K-deficient serum and phenindione serum to be mutually corrective.

TABLE I

ONE-STAGE "PROTHROMBIN" TIME, ASSAYS OF FACTOR VII, AND PROTHROMBIN

	Time after Giving Vitamin K_1, etc. (hr.)	One-stage "Prothrombin" Time (sec.)	Factor VII Assay (%)	Two-stage Prothrombin Assay (%)
Patient (1)	0	27	5	55
	3	22	8	70
	6	20	16	76
	9	18	32	86
	14	16	45	90
	24	14	100	100
	Control	15		
	Phenindione	41		
Patient (2)	0	270	No factor VII present*	16
	24	15	100	100

* The available phenindione plasma had a shorter "one-stage" clotting time than the patient.

Two-stage Prothrombin Assay.—This gave a result 55% of normal.

Effect of Vitamin K.—In Tables I and II are shown the estimations of serial specimens collected at intervals after the administration of 50 mg. vitamin K_1 intravenously. The effect is shown on the one-stage "prothrombin" time, on the prothrombin assay, on the factor VII assay (see Table I), on the serum thromboplastin activity, and on the ability of the serum to correct the serum thromboplastin defect in Christmas disease serum (see Table II).

These results show the lowered concentration of prothrombin and factor VII and the impaired ability of the serum to react in the thromboplastin generating system. The impaired ability to correct the defective serum reaction of Christmas disease serum is also illustrated. The rapid correction of these abnormalities following the administration of vitamin K_1 is demonstrated.

The second patient was a 59-year-old woman known to be suffering from steatorrhoea for three years before the admission during which this study was carried out. She was readmitted on this occasion with extensive spontaneous bruising of the left arm, left shoulder, and over the anterior chest wall.

The bleeding time, tourniquet test, platelet count, and factor V assay were all normal.

The whole blood coagulation time was more than one hour.

Prothrombin Consumption.—There was no coagulation during the hour after withdrawal of the specimen and no utilization of prothrombin during that hour using the technique of Douglas and Biggs (1953). The clotting times obtained from the globulin fractions were as follows:

Normal Plasma	Patient's Plasma	¼ Hour	½ Hour	¾ Hour	1 Hour
15 sec.	92 sec.	98 sec.	90 sec.	86 sec.	97 sec.

TABLE II

OBSERVATIONS ON THE THROMBOPLASTIN GENERATION TECHNIQUE

	Time after Giving Vitamin K_1, etc. (hr.)	Thromboplastin Generation Technique (Serum Component Variable, Clotting Times in sec.)						Thromboplastin Generation Technique (Serum Component 0·2 ml. Christmas + 0·1 ml. Patient, Control, or Christmas, Clotting Times in sec.)					
		1	2	3	4	5	6	1	2	3	4	5	6
Patient (1)	0	55	35	35	32	32	32	57	40	28	30	25	22
	3	62	42	31	28	26	26	35	14	14	16	16	16
	6	45	27	19	16	17	16	22	13	13	14	14	13
	9	60	24	14	13	12	13	17	11	12	12	11	12
	14	52	26	13	12	11	11	13	9	9	9	10	9
	24	50	13	12	10	9	9	13	9	9	9	10	9
	Control	17	9	10	9	9	8	10	9	9	9	9	9
	Christmas							60	45	25	29	25	21
	Phenindione	80	45	52	46	42	45						
	50% 0 hr. 50% phenindione	60	57	42	38	36	31						
Patient (2)	0	43	36	37	37	37	39						
	24	49	13	12	10	10	10						
	Control	38	10	12	11	10	10						
	Christmas	52	48	34	30	31	29						
	50% 0 hr. 50% Christmas	65	45	32	31	33	31						
	50% 24 hr. 50% Christmas	60	27	15	13	12	11						
	50% Normal 50% Christmas	69	28	15	12	11	11						

The one-stage "prothrombin" time was 270 sec. compared with 15 sec. in a control.

Thromboplastin Generation Technique. — The absorbed patient's plasma was normal in the system, but the serum gave a defective reaction (see Table II). The patient's serum was unable to correct the thromboplastin defect in Christmas serum.

Two-stage Prothrombin Assay.—As shown in Table I, there was 16% of prothrombin present.

Effect of Vitamin K.—In Tables I and II it will be seen that before the administration of the vitamin K there was reduced concentration of prothrombin, factor VII, and impaired serum thromboplastin activity and ability to correct the serum thromboplastin defect in Christmas disease. These facets of the defect had been corrected in the specimen examined 24 hours after giving vitamin K_1.

Failure to Correct Recalcification Time of Christmas Disease Plasma.—The impaired ability of this patient's plasma (before giving vitamin K_1) to correct the prolonged recalcification time of Christmas disease plasma is demonstrated (Table III). There is normal ability to correct the haemophilic defect.

TABLE III

RECALCIFICATION TIMES OF PLASMA FROM NORMAL VITAMIN-K-DEFICIENT PATIENT (CASE 2), HAEMOPHILIAC, AND CHRISTMAS DISEASE

	Volumes in ml.				
A		0·1	0·09	0·01	0
B		0	0·01	0·09	0·1
A = Vitamin-K deficient		10	6	7	10
B = Christmas					
A = Vitamin-K deficient		12	4	4	7½
B = Haemophiliac					
A = Vitamin-K deficient		13½	4½	3	2
B = Normal					
A = Christmas		12	4	3	2
B = Normal					
A = Haemophiliac		7½	3½	3	2½
B = Normal					

Clotting times to the nearest half-minute.

Failure to Correct Defective Prothrombin Consumption of Recalcified Christmas Disease Plasma.—The results are shown in Table IV. It will be seen that in comparison with the normal there was very considerable impairment in the ability of the vitamin-K deficient plasma to correct the defective prothrombin utilization of Christmas disease plasma.

TABLE IV

PROTHROMBIN CONSUMPTION OF RECALCIFIED CHRISTMAS PLASMA WITH ADDITIONS OF NORMAL AND PATIENT (2) PLASMA

Christmas Plasma	Incubation Time (sec.)	5′	10′	15′	20′	25′	30′	35′	40′	45′	50′	55′	60′
+Normal	45	10	45	65	61	87	88	104	117	96	96	92	104
	90	12	63	81	69	94	94	113	141	122	117	110	117
+Vitamin-K deficient	45	11	9	10	11	15	16	16	15	17	23	20	19
	90	14	11	11	14	19	20	22	20	19	26	23	24
+Christmas	45	12	12	10	10	13	12	14	12	13	19	14	19
	90	14	15	14	11	16	14	17	14	16	21	18	26

Clotting times of fibrinogen in seconds.

Discussion

This investigation has confirmed the prothrombin-factor-VII defect in vitamin K deficiency. The thromboplastin defect has been studied in some detail. There is a defective serum reaction in vitamin K deficiency on the thromboplastin generation test and failure of mutual correction with Christmas disease serum in this test system. Since serum is an end-product of the coagulation process, this failure of mutual correction could theoretically represent a common defect reached through different channels. New evidence has been produced in this investigation to show that the failure of the two sera to correct is indeed due to a Christmas factor defect in the vitamin-K-deficient blood. The failure adequately to correct the prolonged recalcification time of Christmas disease plasma gives some support to the concept. The failure of the vitamin-K-deficient plasma to correct the defective prothrombin consumption of recalcified Christmas disease plasma is convincing evidence. The results of this test cannot be due to the prothrombin deficiency of the vitamin-K-deficient plasma, since the addition of normal plasma with a normal prothrombin content to the test system results in less residual prothrombin. Since recent evidence indicates that factor VII is not concerned in blood thromboplastin formation, and since the Christmas plasma has a normal content of factor VII, the results cannot reasonably be attributed to the difference in the content of factor VII of the addition.

The administration of vitamin K corrects all the facets of the defect, namely, prothrombin deficiency, factor VII deficiency, the serum thromboplastin defect, and the ability of the serum to correct the thromboplastin defect in Christmas disease serum. The rate of correction of each of these properties is essentially similar. Prothrombin, factor VII, and the Christmas factor have many properties in common. They are all adsorbed by inorganic precipitates, are all deficient in coumarin therapy (Naeye, 1956 ; Sise, Kimball, and Adamis, 1955 ; Douglas and Mair, 1957), and by this study are shown all to be reduced in concentration in vitamin K deficiency, responding at similar rates to the administration of vitamin K. The possibility is further raised that these three components may be more closely identified than is generally envisaged.

The defects produced by vitamin K deficiency and that produced by the coumarin drugs have identical features.

Telfer, Denson, and Wright (1956) and Hougie, Barrow, and Graham (1957) have described cases of constitutional haemorrhagic disease with a prolonged one-stage test and defective serum reaction in the thromboplastin generation system. These abnormal laboratory findings are believed not to be attributable to deficiencies of factor VII or the Christmas factor and have been called respectively the Prower defect (Telfer *et al.*, 1956) and the Stuart defect (Hougie *et al.*, 1957). Since neither of these two defects corrects the coumarin defect adequately, it is possible that the interpretation of the experimental phenomena described in this paper is incomplete.

Summary

Evidence is described indicating a deficiency of the Christmas factor as part of the coagulation defect resulting from lack of vitamin K. This deficiency of Christmas factor has been demonstrated by the impaired ability of vitamin-K-deficient plasma to correct the defective prothrombin consumption of recalcified Christmas disease plasma. Also ability to correct the prolonged recalcification time of Christmas disease plasma and the serum thromboplastin defect in Christmas disease is impaired.

The reduced concentration of prothrombin and factor VII in vitamin K deficiency is confirmed.

The facets of the vitamin K defect (prothrombin, factor VII, serum thromboplastin defect, and the inability of serum to correct the Christmas serum thromboplastin defect) are the same as those resulting from the administration of the coumarin drugs. These components of the vitamin K defect all respond at similar rates to the administration of vitamin K.

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THE ABSENCE OF ABNORMAL HAEMOGLOBINS IN 500 OUT-PATIENTS IN BRITAIN

BY

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The purpose of this investigation was to discover whether abnormal haemoglobins occur among the inhabitants of the British Isles, and, if so, to estimate their incidence. In view of the possible association between endemic malaria and the haemoglobinopathies, abnormal haemoglobins might well occur, malaria being previously endemic in parts of Britain.

The occurrence of haemoglobin D has been reported in persons of partly British descent. It was found in a white North American family of partly Irish and English descent (Itano, 1951), again in a British family with Austrian and Spanish connexions (White and Beaven, 1954), and in a mulatto family where it was inherited from an English mother with no known foreign antecedents (Stewart and MacIver, 1956).

The sample investigated comprised 500 outpatients of St. Bartholomew's Hospital, London, referred for investigations requiring venous blood. The bearers of names judged not native to the British Isles were excluded. It was assumed that this group would constitute a fair sample of the British-born population of the four home countries.

The method of investigation used was paper electrophoresis, using veronal buffer at *p*H 8.6 which would detect haemoglobins A, C, S/D, E, G, H/I, J, K, and L. Suspicious slowing of the normal adult haemoglobin (A) band was followed by testing for the presence of foetal haemoglobin (F) using the standard one-minute alkali denaturation test (Singer, Chernoff, and Singer, 1951).

Details of the electrophoretic technique are given by Lehmann and Smith (1954).

Results

The 500 cases examined yielded no abnormal electrophoretic patterns. All haemoglobins moved as a single band in the position of normal adult controls (AA pattern), and in no case was the foetal haemoglobin found to be abnormal.

Conclusion

It is possible that families with inherited abnormal haemoglobins exist in the more remote and isolated parts of the British Isles and would be detected in larger samples or samples from particular regions. However, none was found in this series. Thus abnormal haemoglobins either do not occur or are rare among the inhabitants of Great Britain.

I should like to thank Dr. H. Lehmann for suggesting this investigation.

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FATAL *LISTERIA MONOCYTOGENES* MENINGITIS IN A CHILD AGED 3 MONTHS

BY

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Listeria infections are common amongst animals, but do not often occur in man.

The case reported here was of a boy aged 3 months, the youngest of three children, who had a birth weight of 6 lb. 12 oz. following an uncomplicated pregnancy. No abnormality was detected until one week before admission to hospital, when he became fretful at night, screaming persistently, followed by increasing anorexia and apathy. There was evidence of slight respiratory infection with cough, "snuffles," and feverishness. No skin rashes were observed. He had a major convulsion the night before admission.

On admission on August 8, 1956, he had a temperature of 103° F. and was moderately dehydrated. There was a mucoid discharge from both nostrils. No moist sounds were heard in the chest. The anterior fontanelle was tense and bulging and there was some neck stiffness. Kernig's sign was positive on both sides.

Direct examination of a specimen of cerebrospinal fluid taken on admission showed that numerous polymorph leucocytes were present, but no organisms were seen in a film from the centrifuged deposit. Culture yielded a scanty growth of a small Gram-positive bacillus; this was thought to be a "diphtheroid" organism, and on account of the very small number of colonies was regarded as a contaminant. Blood culture at this stage was negative.

The child was treated as follows for nine days: intrathecal penicillin, 10,000 units daily for three days; intramuscular penicillin (soluble), 500,000 units at once, followed by 200,000 units six hourly for five days, and distaquaine penicillin, 150,000 units daily for a further four days; sulphadimidine 1 g. at once followed by 0.25 g. six hourly for nine days. On the third day of treatment a second specimen of cerebrospinal fluid was examined and found to contain polymorph leucocytes; there was no growth on culture. A small ulcer from which *Proteus* was the only organism isolated developed on the left buttock at the site of the first penicillin injection.

On admission the white cell count was 16,000 cells per c.mm. (88% polymorphs, 11% lymphocytes, and 1% monocytes). Five days later there were 8,800 leucocytes (67% polymorphs, 2% eosinophils, 30% lymphocytes, and 1% monocytes). The temperature fell to normal and recovery was apparently complete.

On August 23, 1956, the fever recurred. He was treated with soluble penicillin, 250,000 units six hourly, and sulphadimidine, 0.5 g. six hourly for three days, and subsequently with chloramphenicol, 100 mg. six hourly, without response. Signs of meningitis reappeared and successive lumbar punctures at this stage showed the following appearances in the cerebrospinal fluid (centrifuged deposits): August 28, occasional red blood cells only; August 30, a few leucocytes, mainly lymphocytes; August 31, numerous polymorph leucocytes and a few lymphocytes.

From this last specimen a Gram-positive bacillus was isolated similar in all respects to that cultured from the cerebrospinal fluid on admission. A blood culture taken on August 28 was contaminated, but similar Gram-positive bacilli were found in the mixed growth obtained.

The organism was provisionally identified as *Listeria monocytogenes* and the treatment was changed to intramuscular streptomycin, 250 mg. b.d., and oral erythromycin, 70 mg. six hourly, in accordance with the sensitivity findings (see bacteriological data). The treatment was, however, ineffective and the child died on September 5, 1956.

During the last few days of life specimens of cerebrospinal fluid were examined daily and showed a marked polymorphonuclear reaction with occasional lymphocytes only; on each occasion a heavy growth of *List. monocytogenes* was obtained.

In view of these findings the family history is of particular interest. Both parents were healthy though the mother had been blind since birth. The father worked as a soil analyst, but had noted nothing abnormal either in his work routine or his personal health at the time of his son's illness. During the first week of the child's illness the second child had a mild respiratory infection while the eldest remained well. There are no domestic pets in the family, and the standard of hygiene in the home was excellent.

Necropsy

The post-mortem findings were as follows:

The body was that of a well-nourished male child aged about 3 months. Small, roughly circular red macules were present in the skin over the buttocks and groin. A single small ulcer, about

1 cm. in diameter, having a grey firm base and clean-cut edges, was on the outer aspect of the left buttock.

The brain and spinal cord were everywhere covered by purulent exudate. Over the superior and anterior aspects of both frontal lobes, the inferior aspect of both temporal lobes, and over the base of the brain, the exudate was yellow and of the consistency of thick cream ; elsewhere over the brain and spinal cord it was more translucent and gelatinous with discrete small purulent nodules measuring up to 0.3 cm. diameter scattered throughout.

No pus was seen in either middle ear cavity. The heart, lungs, liver, kidneys, spleen, and other viscera and organs showed no important macroscopic abnormality. Swabs for culture of organisms were taken at necropsy from both middle ear cavities, brain, and spleen.

Brain and Spinal Cord.—The subarachnoid space was filled with chronic purulent inflammatory exudate which had spread along the perivascular spaces into the depths of the sulci. The grey matter of the underlying brain was oedematous and in it were occasional small superficial abscesses. The histological appearances of the exudate were indistinguishable from those seen in any other purulent meningitis. Similar changes were seen in the spinal cord. Sections stained with Gram's method showed cocco-bacillary organisms morphologically indistinguishable from *Listeria monocytogenes* (Fig. 1) ; they appeared to be both extracellular and intracellular, and had variable staining properties.

Spleen and Lymph Nodes.—The general architecture was preserved, but Malpighian bodies and lymphoid follicles were small and composed entirely of deeply-staining small lymphocytes. There was moderate generalized fibrosis throughout the pulp, which in the spleen was moderately congested and contained numerous macrophages. Polymorphonuclear leucocytes were not present, and appearances were those of chronic inflammation with fibrosis.

Sections from the heart, lungs, liver, pancreas, kidneys, and adrenals showed no important abnormality.

Gram-staining of sections other than those of brain and spinal cord failed to reveal any organisms.

Bacteriology

The characteristics of the *Listeria* isolated from this case were as follows:

The organism was a small Gram-positive but

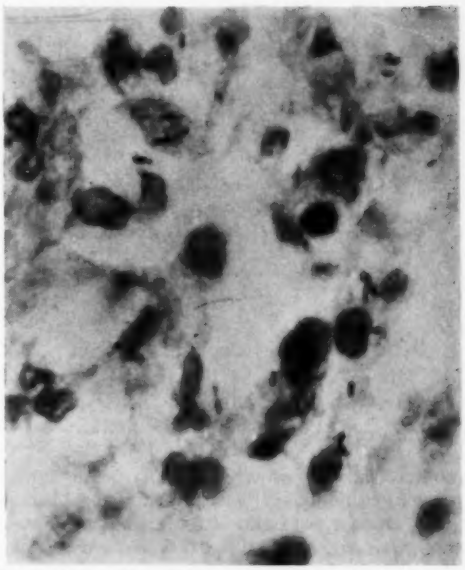

FIG. 1.—Gram stain × 960 to show *Listeria* organisms in the meningeal exudate.

easily decolorized bacillus occurring singly or in pairs with occasional short chains only.

After overnight cultivation in broth at 37° C. the organism was non-motile, but when grown at 22° C. it showed a vigorous " tumbling " motility.

Growth in the form of small, translucent, smooth colonies was good at 37° C. and slower at 4° C., on ordinary nutrient agar. Growth also occurred on McLeod's tellurite agar, on McConkey's medium, and in 6% sodium chloride broth. On 5% fresh horse blood agar colonies were surrounded by a narrow zone of haemolysis. The catalase test was strongly positive and the oxidase test was negative. There was no liquefaction of gelatin or of Loeffler's serum.

Biochemical Tests.—The methyl-red and Voges-Proskauer tests were positive, there was no growth in citrate medium, and no H_2S or indole was produced.

The organism fermented, with the production of acid but not gas, aesculin, glucose, lactose, laevulose, maltose, mannose, rhamnose, salicin, sucrose, and trehalose, and failed to ferment arabinose, dulcitol, galactose, inositol, inulin, mannitol, raffinose, sorbitol, and xylose.

Conjunctival Test.—Confirmation that the organism was a *Listeria* was obtained by instilling two drops of an overnight broth culture into the

FIG. 2.—Conjunctival test in guinea-pig to show marked corneal opacity.

conjunctival sac of a guinea-pig. An intense purulent conjunctivitis was present after 48 hours (from which a pure and heavy growth of *Listeria* was obtained), but this quickly resolved leaving after five days a marked corneal opacity (Fig. 2). This gradually disappeared and after three weeks the eye appeared normal. It was found, as reported by Julianelle (1941), that the eye subsequently resisted reinfection. This immunity did not, however, extend to the contralateral eye in which a similar reaction could still be demonstrated.

Serology.—The organism was sent to Dr. H. Seeliger, of Bonn, for serological typing, and he reported that it belonged to serotype 4b.

The organism was sensitive to streptomycin, erythromycin, and oleandomycin, weakly sensitive to achromycin, aureomycin, sulphadiazine, sulphanilamide, and sulphadimidine, and resistant to terramycin, chloramphenicol, novobiocin, and penicillin.

Discussion

List. monocytogenes is an uncommon cause of meningitis in man. Seven cases have now been reported in the British literature (Gibson, 1935; Wright and Macgregor, 1939; Barrow and Pugh, 1957; Edmunds, Nicholson, and Douglas, 1957; Mair, Mair, Stirk, and Reid, 1957). In a review of the world literature, Finegold, Bradley, Campbell, and Greenberg (1954) listed 26 cases certainly and 11 cases possibly due to *List. monocytogenes* and described a further two cases of their own. Recently, however, further cases have been reported from Germany (Eck, 1957) and the U.S.A. (Dedrick, 1957), and the disease may, in fact, be more common than has been generally supposed.

The disease usually presents as an acute meningitis and responsibility for the precise diagnosis rests with the pathologist. Examination of the cerebrospinal fluid reveals a marked polymorphonuclear reaction (the infection is not usually associated with a monocytic response in man) and in many cases small bacilli. Because the organism is easily overdecolorized it may appear to be Gram-negative and at this stage may be mistaken for *Haemophilus influenzae*. On culture, however, the ability of the organism to grow abundantly on simple nutrient agar eliminates this possibility.

Mathieu, Young, Mennillo, and Sherwood (1956) suggested that the colonial and morphological appearances of *List. monocytogenes* might lead to the organism being regarded as a streptococcus or a diphtheroid contaminant. The latter possibility is much the more likely and further tests are necessary to identify the organism. The most significant of these are (*a*) the motility test, when, if the organism is grown in broth at 22° C., a vigorous "tumbling" motility is observed; (*b*) growth in 6% salt broth, when the organism grows readily in a fluid medium in the presence of 6% NaCl; (*c*) a conjunctival test, when a typical conjunctivitis and subsequent keratitis occurs after instillation of a broth culture of the organism into the conjunctival sac of guinea-pig or rabbit.

The findings in the central nervous system in this child, as in the fatal cases reported by Gibson (1935), Burn (1936), Wright and Macgregor (1939), and others (see review by Finegold *et al.*, 1954) are those of a non-specific purulent meningitis. Lesions in the liver, such as have been reported by Wright and Macgregor, are absent. Fibrosis of the splenic pulp has not been reported in any of the fatal cases reviewed by Finegold *et al.*; it is probably the end-result of chronic inflammation and is presumably due to *Listeria* infection, though no organisms can be demonstrated in the sections.

The source of *Listeria* infection in human cases is rarely discovered. There is no evidence that the disease is transmitted directly from animals, and the consensus of opinion is that in babies the most likely source of infection is the maternal genito-urinary tract. The high proportion of neonates amongst cases of *List. monocytogenes*

meningitis appears to support this view (Girard and Gavin, 1957). The case reported by Barrow and Pugh (1957) was a premature baby 12 days old, where a "diphtheroid" organism, not precisely identified, had been recovered previously from the mother's urine. It is interesting to note that the child was delivered by Caesarean section, so that transmission of infection from the mother, if it occurred, was probably *in utero*. An investigation into the frequency with which *List. monocytogenes* occurs in the female genito-urinary tract, especially during pregnancy, might throw some light on the origin of meningitis due to this organism.

Summary

Clinical, bacteriological, and histological findings from a fatal case of *Listerella monocytogenes* meningitis are reported.

Responsibility for the diagnosis of this disease rests with the pathologist, and a comment is made on the most useful tests to establish the identity of organisms.

Our thanks are due to Professor W. S. Craig for permission to publish details of the case, and to Dr. H. Seeliger, of Bonn, for serotyping the organism.

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THE SENSITIVITY *IN VITRO* OF THE PROVIDENCE GROUP OF ENTERIC BACTERIA TO 14 ANTIBIOTICS AND NITROFURANTOIN

BY

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During the course of an investigation into the antibiotic sensitivity of strains of the *Proteus* group of bacteria isolated from infections of the urinary tract (Middleton, 1957) a few strains of the Providence group were also examined. This group of the Enterobacteriaceae called type 29911 by Stuart, Wheeler, Rustigian, and Zimmerman (1943), and named Providence by Kauffmann (1951), has close affinities in many characteristics with the *Proteus* group (Singer and Bar-Chay, 1954), and a similarity between strains of the two groups was found here in their common resistance to many antibiotics *in vitro*. An opportunity has since arisen to study a larger number of Providence strains and a relationship has also been demonstrated between the biochemical types of Providence strains and their antibiotic spectra.

Material and Methods

Forty Providence strains were examined. Nine were isolated in this laboratory, of which eight were from urine and one from faeces. The 31 other strains were obtained from Dr. K. P. Carpenter, Director of the Dysentery Reference Laboratory, Central Public Health Laboratory, Colindale, London. These were recently isolated organisms which had been sent there from various centres in Britain and the Commonwealth; one was from urine, 29 from faeces, and the source of one strain was unknown.

The identification of the strains isolated in this laboratory was based on the criteria described by Kauffmann (1954). This identity was kindly confirmed by Dr. Carpenter, who, in addition, classified these strains, and those supplied by her, into the biochemical subdivisions described by Ewing, Tanner, and Dennard (1954).

The dried disc technique described by Fairbrother and Jennings (1955) was used for testing the sensitivity of the strains to penicillin (2.5 units), novobiocin (20 μg.), bacitracin (10 u.), erythromycin (10 μg.), spiramycin (60 μg.), oleandomycin (10 μg.), streptomycin (80 μg.), neomycin (50 μg.), soframycin (50 μg.), tetracycline (100 μg.), chlortetracycline (100 μg.), oxytetracycline (100 μg.), chloramphenicol (100 μg.), polymyxin (500 u.), and nitrofurantoin 10,000 μg.). (The concentration of each drug per paper disc or test tablet is given in brackets.) A zone of inhibition of growth 10 mm. or more in diameter was considered to indicate sensitivity to the antibiotics, the discs or test tablets being 5 mm. in diameter. Any inhibition of growth round the nitrofurantoin test tablet was taken to indicate that the organism was sensitive to the drug.

A few strains were also tested by the serial dilution technique against streptomycin, tetracycline, and chloramphenicol. A standard drop (0.02 ml.) of a 1/10,000 dilution of an overnight broth culture was added to a series of tubes containing 1 ml. of nutrient broth plus doubling dilutions of each of the antibiotics separately. These results, as also those of the disc tests, were read after about 18 hours' incubation at 37° C.

Results

The biochemical classification of the 40 Providence strains is shown in Table I. All the strains isolated from urinary infections were of Ewing's biotype 27.

TABLE I

BIOCHEMICAL CLASSIFICATION OF 40 PROVIDENCE STRAINS

Ewing, Tanner, and Dennard Biochemical Group	No. of Strains	Ewing, Tanner, and Dennard Biotype	No. of Strains
1	20	1	5
		4	4
		5	7
		6	4
2	20	21	7
		27	13

All the 40 strains were resistant to penicillin, novobiocin, bacitracin, erythromycin, spiramycin, and oleandomycin. All were sensitive to neomycin, soframycin, and nitrofurantoin. Fifteen per cent. of strains belonging to Ewing's biochemical group 1 and 10% of Ewing's bio-

TABLE II
BACTERIAL SENSITIVITY *IN VITRO* OF 40 PROVIDENCE STRAINS (DISC TEST)

Organism	No. of Strains Tested	Percentage Sensitive to Certain Antibiotics and Average Diameter of Zone of Inhibition of Growth (mm.) of All Strains				
		Streptomycin	Tetracycline	Chlortetracycline	Oxytetracycline	Chloramphenicol
Biochemical Group 1 ..	20	100 11·7 (10–14)*	45 9·6 (7–15)	15 8·7 (6–13)	75 11·0 (6–16)	100 15·6 (11–20)
,, ,, 2 ..	20	10 6·7 (5–11)	0 6·4 (5–8)	0 6·5 (5–8)	10 7·1 (5–12)	15 7·8 (5–11)

* Limits of inhibition of growth in brackets. Disc diameter 5 mm.

chemical group 2 strains were sensitive to polymyxin. With streptomycin, the tetracycline antibiotics, and chloramphenicol a difference in sensitivity between strains of biochemical groups 1 and 2 was found, as is shown in Table II. Strains of the former group were mainly sensitive and those of the latter group resistant to these antibiotics. There was no marked variation from this general pattern with individual strains of the biotypes composing these biochemical groups, except that all biotype 1 strains tested were resistant to the tetracycline analogues; biotype 21 strains were all uniformly resistant to streptomycin, the tetracycline drugs, and chloramphenicol. The average diameters, and the limits, of the zones of inhibition of growth found in the disc tests, which were also recorded in Table II, show that the difference in sensitivity between the two biochemical groups was marked. Relatively large zones of inhibition of growth were obtained with biochemical group 1 strains, whereas with biochemical group 2 strains either no inhibition of growth occurred or only small zones were produced. This difference was confirmed by the results of the serial dilution tests (Table III), and was more than 16-fold with each antibiotic.

TABLE III
BACTERIAL SENSITIVITY *IN VITRO* (TEST TUBE)

Providence Strain (Typical)		Minimum Inhibitory Concentration of Antibiotic (μg./ml.)		
		Streptomycin	Tetracycline	Chloramphenicol
Biochemical Group 1	Biotype 6	31	3·9	7·8
Biochemical Group 2	,, 21	>500	>500	250
	,, 27	>500	250	250

Discussion

Brooke (1951) tested the antibiotic sensitivity of 35 Providence strains isolated from urine. These were all resistant to penicillin and sulphathiazole, and most of the strains were resistant or only slightly sensitive to streptomycin, chlortetracycline, oxytetracycline, and chloramphenicol. Biochemically these strains resemble strains of Ewing's biochemical group 2 and especially biotype 21, although from the details given by Brooke it is not possible to reclassify the organisms precisely under Ewing's system. Lutz and Hofferer (1955) examined 16 strains; all were sensitive to neomycin and soframycin, and 11 were sensitive to chloramphenicol, but all were resistant to streptomycin and the tetracycline antibiotics. Biochemically 14 of these strains correspond to Ewing's biotype 4 and the other two to biotype 27. The findings of the present investigation are in general agreement with these results, except that the four strains of biotype 4 which were tested here were more sensitive to streptomycin and the tetracycline drugs than those of Lutz and Hofferer (1955).

Lutz and Hofferer showed that Providence strains as a whole have a different antibiotic spectrum from that of the four *Proteus* species, each of which has a relatively distinct pattern of sensitivity to antibiotics, as has been found by these authors and Poole (1954) and Potee, Wright, and Finland (1954). This has in general been confirmed, but if the antibiotic spectra of the strains of Ewing's biochemical groups 1 and 2 are considered separately they show some resemblance to the antibiotic spectra of strains of *P. morganii* and *P. rettgeri*. Strains of these *Proteus* species are more often either uniformly sensitive or resistant to streptomycin, the tetracycline antibiotics, and chloramphenicol than those of *P. mirabilis* and *P. vulgaris*. There is also a very close similarity between them and Providence strains in their susceptibility to other antibiotics, and it is these *Proteus* species which Providence is most like in other respects. Providence strains are not infrequently confused with the shigellae, and biochemical group 1 strains have an antibiotic spectrum similar to that of the latter genus. However, *Proteus* and Providence are generally resistant to polymyxin, whereas the shigellae are very sensitive to this antibiotic as was shown by

Felsenfeld, Volini, Ishihara, Bachman, and Young (1950).

It is recognized that strains from only six of the 31 biotypes described by Ewing *et al.* (1954) have been studied, but, since these six biotypes comprised 71% and 62.7% respectively of strains of the biochemical groups 1 and 2 formulated by these authors during their study of 611 strains, the results are considered to be reasonably representative.

It is interesting that all the nine strains isolated from urine belonged to biotype 27. It is possible that some of the eight found in this laboratory were examples of cross-infection, but it is unlikely that they all were, since they were isolated over a period of a number of years and some were from different hospitals. The 35 strains from urine reported by Brooke (1951) were also probably of biochemical group 2. The biochemical classification of the 15 strains from urine which were studied by Ewing *et al.* (1954) is not given by the authors, but from the details recorded of their antigenic grouping it is possible that these strains also were of biochemical group 2. On the other hand the four strains isolated from urine by Lutz and Hofferer (1955) were equally divided between the two biochemical groups. However, since only 13% of the 611 Providence strains, from all sources, examined by Ewing *et al.* (1954) were of biochemical group 2, and only one was biotype 27, the above findings suggest a predilection of organisms of this biochemical group for the urinary tract.

On the basis of the sensitivity tests *in vitro* there is as yet a paucity of chemotherapeutic agents available for use in infections due to some Providence strains, and especially in systemic and urinary infections. This may be in part the explanation of the more frequent isolation of the most resistant strains from the urine. All the cases observed here had infections complicated by other disease of the urinary tract, and it is such cases which, as a result of prophylactic or repeated chemotherapy, often become infected with resistant organisms.

Summary

The sensitivity *in vitro* of 40 strains of the Providence group of enteric bacteria to 14 antibiotics and nitrofurantoin has been determined.

The strains had been classified biochemically into the biochemical groups and biotypes described by Ewing *et al.* (1954).

The individuality of the antibiotic spectrum of this group as a whole has been confirmed, but strains of the biochemical groups 1 and 2 differed in their sensitivity to some antibiotics and considered separately showed a resemblance to strains of *P. morganii* and *P. rettgeri* in this respect.

All the strains isolated from urine were of biochemical group 2. A similar trend in the predilection of strains of this biochemical group for the urinary tract has been found on examining the reports of other authors.

There is as yet a paucity of chemotherapeutic agents available for use in infections due to some Providence strains, and especially in systemic and urinary infections.

I am most grateful to Dr. K. P. Carpenter for providing most of the organisms examined and for classifying them biochemically.

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TECHNICAL METHODS

The Sharpening of Microtome Knives

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The regular production of a satisfactory edge on a microtome knife for the cutting of sections as thin as 2 μ, which are free from scores and other blemishes, is a serious technical problem in histological laboratories. Even when a good edge is produced on a knife, its life is generally limited because contact with any hard material in the specimen may damage the delicate edge, causing it to score subsequent sections.

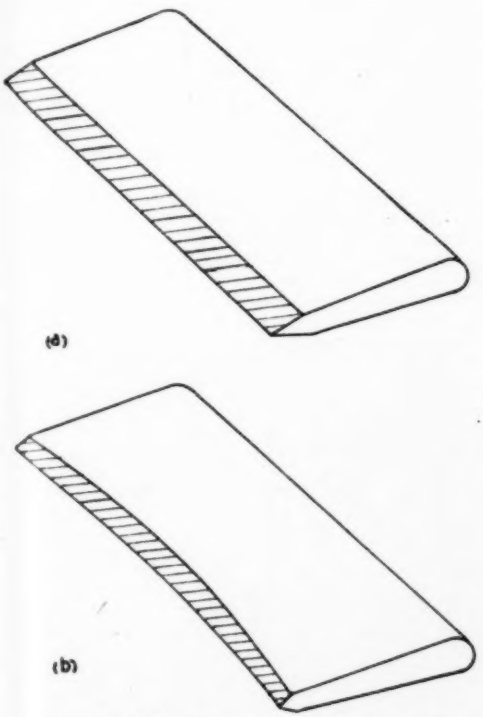


FIG. 1.—(*a*) Straight edge formed by intersection of flat facets. (*b*) Curved edge formed by intersection of concave facets.

A knife edge is formed by the intersection at an appropriate angle of two smooth surfaces (Fig. 1). It is well known that if the surfaces are flat, the edge formed by their intersection will be straight; if they are not flat, the edge will be curved. If the surfaces are not smooth, the edge formed by their intersection will be ragged and unsuitable for fine work.

The procedure described in this paper has been developed by applying to the preparation of the facets forming the edge techniques used for the production on steel of optically flat surfaces having a surface roughness of the order of 0.01 μ.

Conventional Methods of Sharpening

The hand sharpening technique which has been used for many years may be described as follows.

A cylindrical metal back of the same length as the knife is clamped to the back of the blade, and the handle is fixed to one end of the blade. The knife is laid on a fine hone, liberally coated with water, and then moved, with the edge leading, over the surface of the hone under a very light pressure. The purpose of the metal back on the knife during sharpening is to produce the desired angle on each of the facets forming the edge.

If the bearing surfaces on the back lie in a plane through the knife edge, it will be possible to produce a flat facet. In general, however, this edge will not be parallel with the bearing surfaces on the back, and if, during sharpening, it is lying along the surface of the hone, the back will be in contact with only one edge of the hone and a facet of varying angle will be produced. Further, as the length of the blade is generally greater than the width of the hone, more work will be done on the central zone of each facet and concave facets and a curved edge will result.

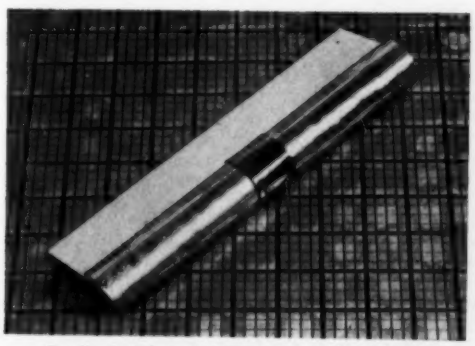


FIG. 2.—Experimental sharpening of a knife by lapping.

When it is considered that a suitable finish has been achieved on each facet, and the edge is free from gaps, the knife is given a few very light strokes over a leather strop.

Preparation of Knives by Lapping

Preliminary Experiments.—When the problem was first examined it became clear that the first step would have to be to develop some better means for locating the knife on the sharpening medium than that achieved by the conventional type of back. If a knife is placed on a flat surface and the back of the knife is supported close to its midpoint, then the edge of the blade will lie evenly on the flat surface. To secure this geometry a narrow band of hard steel was fixed to the middle of the conventional back which was clamped to the knife (Fig. 2). Then, when the knife was placed on a flat surface, it rested, in general, at the midpoint of the back and at two points on the edge, unless the form of the facet happened to match exactly the contour of the surface.

The knife, mounted in this way, was sharpened by lapping on a flat bronze plate impregnated with fine abrasive powder. The surface of the plate is chequered by two sets of parallel grooves at right angles to each other. Their purpose is to prevent the accumulation of debris on the surface and to stop the formation of a "wire edge."

The lapping plate is liberally wetted with a suspension of abrasive (Al_2O_3) in a suitable medium (see Appendix), and the knife in its holder is worked over the plate with a circular movement, care being taken to avoid bringing the edge parallel with either set of grooves. As the knife is moved over the plate, metal is abraded off those parts of the blade (and the back) which are in contact with the surface of the lap. As the work continues, a point is reached where the whole of the lower facet of the knife is in contact with the plate and metal is being removed uniformly from all parts of this facet. The knife is turned over and the same process repeated on the second facet. During this initial sharpening process it may be necessary to add further abrasive suspension to the plate.

When this stage has been completed and the lap is cutting evenly over the whole of each facet, the surfaces will present a slightly matt appearance. To produce the fine surface finish on the knife a small quantity of abrasive suspension is poured on to the plate and worked over with a hardened flat steel disc, which pushes the tiny grains of abrasive into the soft surface of the bronze. After about two minutes' work with the steel block, the plate is flooded with a solution of detergent in water and scrubbed with a stiff brush to remove all loose particles of debris and abrasive. The plate is rinsed several times with tap water and dried with absorbent paper.

To complete the sharpening process, the plate is wetted with a suitable lubricant (see Appendix) and the knife worked over the plate exactly as before. An examination of the facet after only a very short period of lapping (two or three minutes) shows a beautiful specularly reflecting surface developing on the facet as the scratches due to the loose abrasive are removed. Each facet is lapped in this manner and the final finish is obtained by drawing the knife across the plate about six times with a straight stroke at right angles to the blade with the edge trailing.

Each of the facets formed in this way is accurately flat and the edge is very straight. In a blade of good quality steel, a microscopic examination of the edge at a magnification of 100 generally fails to reveal any imperfections, and at a magnification of 400 very few imperfections and departures from straightness can be detected.

When this method is employed, the initial sharpening of a used knife with curved facets may take two or three hours, but the resharpening of even a seriously damaged blade takes less than 30 minutes, providing that the back is correctly placed on the knife.

Final Form of Apparatus.—During the preliminary experiments several disadvantages became apparent.

The most serious was the behaviour of the band of hard metal fixed to the back of the knife. This band failed to give a completely satisfactory location of the blade and to determine the angle of the facet. Metal was, of course, removed from this supporting band and in time it had to be replaced. Also, from the nature of the support, it was impossible to vary the angles on the facets. It was also found in lapping a stellite knife that material was removed from the support more rapidly than from the blade, with the result that a good edge could not, therefore, be obtained.

The fixture finally developed for holding knives for sharpening consists of a steel block with a tapered slot carrying the knife which is locked into the fixture by means of two screws bearing against the back of the knife. The feet of the fixture, which are in contact with the lapping plate, are each made of a tungsten carbide pad fixed to a self-aligning bearing which is fixed to the end of a screw passing through the fixture. Tungsten carbide is a very hard material which is not lapped away by the abrasive used for sharpening the knives, so troubles due to wear of the supports are eliminated. The use of a self-aligning bearing to hold each pad ensures that the surface of the pad lies flat on the surface of the lapping plate. The angle of each facet is controlled independently by the adjusting screws, each of which is fixed in position during lapping by means of a lock nut. The dimensions of the experimental fixture are such that the facet angles can be continuously varied from about 12½° to 20°, corresponding to cutting angles of 25° to 40°.

The use of a fixture giving a controllable facet angle greatly extends the useful life of a blade and facilitates resharpening. When a knife is sharpened initially the jig is set to give a minimum cutting angle. Each time the knife is resharpened with the same cutting angle the width of the facet is increased, as is

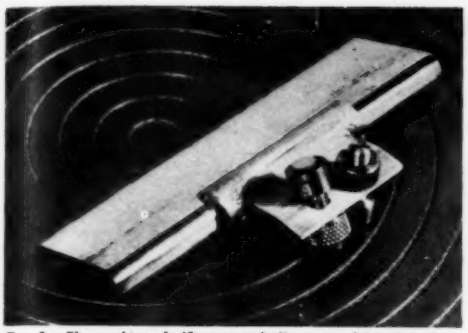


FIG. 3.—Sharpening a knife on a spirally grooved lapping plate.

also the amount of material which must be removed from the facet to obtain a sharp edge. When a stage is reached where sharpening takes an unduly long time, the facet angles are increased by advancing each adjusting screw by about half a turn so that on lapping a new pair of narrow facets is produced and the sharpening time kept within reasonable limits.

The second modification was made to the lapping plate. It is essential for the production of a high quality flat facet that the surface of the lap should be grooved. For most of the work for which these laps are used, two sets of straight parallel grooves at right angles are quite satisfactory. However, when a knife with a narrow facet is worked on such a lap, disastrous results will follow if, during lapping, the edge becomes aligned with either set of grooves. The form of grooving adopted was spiral in the surface, having a width and depth of $\frac{1}{16}$ in. and a pitch of $\frac{1}{4}$ in. The main reason for choosing this form of grooving is that it is easy to produce in a lathe; concentric circular grooves are also satisfactory.

A knife mounted in the fixture and resting on a spirally grooved plate is shown in Fig. 3.

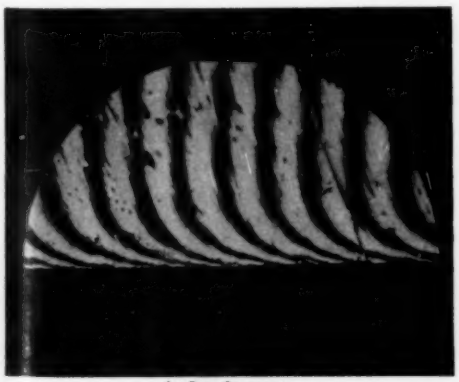
Results

Two entirely different methods were used for assessing the quality of a knife. In the first, the knife was examined under a high-power microscope and a search made for any defects in the edge. The facets were also examined under an interference microscope (Linnik type, by Hilger & Watts) which gave a good assessment of the surface texture or roughness and of the flatness of the facet in the immediate neighbourhood (within 0.002 millimetre) of the actual edge.

In the second method the knife was used to cut serial sections of a variety of types and its quality estimated by the number of satisfactory sections which were cut.

The results of the microscopic examination are shown in Figs. 4 to 7. Each figure comprises an interferogram (*a*) and a photomicrograph (*b*) using vertical illumination. In all the interference photographs the interference bands are closely at right angles to the edge. The dark bands are "contour lines" of the surface of the facet, the contour interval being 0.25 μ. Thus, if the surface is perfectly flat and free from imperfections, the bands would be smooth and straight. Surface imperfections show up as irregularities in the bands, and departures from flatness of the facet are shown by curvature of the bands.

Fig. 4 shows a facet of a knife sharpened in the conventional manner on a fine hone followed by very light stropping on canvas impregnated with abrasive powder, and finally on a leather strop mounted on a wooden bat. The interference bands are straight to within a short distance, about 0.03 mm. of the edge, and then curve off smoothly by about 0.5 μ, showing a distinct rounding of the edge. The surface of the metal is fairly smooth, but shows a characteristic smeared appearance where the stropping has flawed the surface of the metal. The edge, however, is straight, free from "nicks" and gaps, and should be suitable for cutting sections.



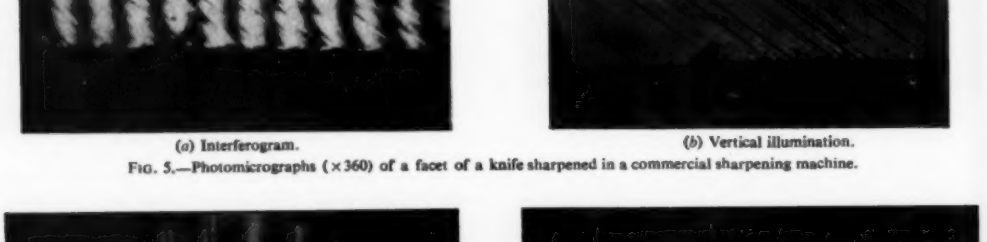
(*a*) Interferogram.

(*b*) Vertical illumination.

FIG. 4.—Photomicrographs (×360) of a honed and stropped facet of a knife.

(*a*) Interferogram. (*b*) Vertical illumination.

FIG. 5.—Photomicrographs (×360) of a facet of a knife sharpened in a commercial sharpening machine.



(*a*) Interferogram. (*b*) Vertical illumination.

FIG. 6.—Photomicrographs (×360) of a lapped facet of a knife.

(*a*) Interferogram. (*b*) Vertical illumination.

FIG. 7.—Photomicrographs (×80) of a knife, showing new and old facets.

A facet of a knife sharpened on a commercial knife-sharpening machine is shown in Fig. 5. It will be noted that the surface is crossed by a number of relatively deep scratches, and that the edge is somewhat ragged but is not perceptibly rounded.

Photographs of facets of knives prepared by lapping are shown in Figs. 6 and 7. The interference bands show no rounding off at the edge in Figs. 6 and 7. In each case the surface of the facet is very smooth and the edge of the knife is free from "nicks" and gaps and is quite straight.

In Fig. 7 is shown the result obtained when the angle of the facet is increased slightly from that originally on the knife. The straight bands normal to the edge (Fig. 7 *a*) are on the new facet and the inclined bands are on the original facet which is heavily scratched. The angle between the new and old facet is approximately 80 minutes and the width of the new facet is approximately 0.1 mm. It is of interest to note that the total time taken for mounting and lapping the knife to form the new edge was less than 10 minutes.

It will be seen from Figs. 6 and 7 that the edges prepared by lapping have all of the desirable features required of a good knife: a minimum of rounding on the edge, freedom from "nicks," and very smooth facets.

The results of the performance tests on these knives may be summarized as follows:

(1) A single portion of a blade was used to cut 850 frozen sections from a number of specimens before any deterioration was noticed.

(2) Another knife was used to cut 550 sections from a specimen mounted in pure celloidin before any deterioration was noted.

(3) The same knife, after resharpening, was used to cut 2,850 paraffin-mounted sections before any deterioration was noted.

(4) In another case a knife, prepared by lapping, was used to cut serial 8μ sections from large specimens of brain embedded in celloidin.

An overall measure of the efficiency of the method is shown by a comparison of the times spent on sharpening in a large hospital laboratory, where approximately four man days per week were spent in preparing knives by conventional methods. The applications of the lapping technique has reduced this time to approximately three man hours.

Summary

Improved techniques and equipment are described for the sharpening of microtome knives. The knife is sharpened by lapping on a grooved flat plate using fixed abrasive. The knife is held in an adjustable fixture which ensures correct presentation of the blade to the lap and the cutting angle is readily adjustable over a wide range. Two methods of assessing the quality of a knife are described, and it is shown that the methods described are quicker than those generally used and produce a better edge.

It is a pleasure to acknowledge the help of a number of colleagues, in particular Mr. J. Lobb and Miss D. R. Colohan, who prepared the majority of knives used in the investigation, and Mr. M. J. Puttock, who operated the interference microscope. Thanks are due to Dr. B. Turner and Mr. V. Conlay, of the Pathology Laboratory, Broughton Hall, N.S.W. Department of Mental Hygiene, who made the practical tests with the knives after they had been sharpened, and to Mr. G. Merritt, of the C.S.I.R.O. Division of Animal Health and Production.

APPENDIX

A suitable lubricant for lapping work consists of an emulsion of

Mineral white spirit	400 ml.
Water	1,000 ml.
Ethyl (or power) alcohol	1,000 ml.
Turkey red oil	45 ml.

This is not a permanent emulsion and must be well shaken before use.

The abrasive suspension is prepared by adding approximately 2 ml. of abrasive powder to about 50 ml. of the emulsion. The abrasive is well graded aluminium oxide grain 4μ diameter.

If, in warm weather, the lapping plate tends to become sticky due to the rapid evaporation of the volatile components, a small amount of white spirit should be added to the plate during lapping to enable the work to be moved smoothly over the plate.

A New Technique for Testing Antibiotic Sensitivity

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The method described below is simple and could easily be used in a routine clinical laboratory. It would also be of value for the investigator who wants to know (*a*) the quantitative difference between the effects of various concentrations of the same antibiotic ; (*b*) the qualitative difference between the same amounts of different antibiotics ; or (*c*) the effects of antibiotics on the growth phase and metabolism of certain organisms.

Method

Apparatus.—The apparatus used for redox potential measurement is the Jones *p*H meter. Readings are taken on the cultures incubated in a thermostat at a desired temperature at intervals set by the experimenter. Either several cultures are used and then at each interval a new culture is tested, or one culture is used throughout and then a sample is taken out under sterile conditions at each interval. In these trials both methods gave similar results and in general the one-tube technique was adopted in the experiments.

Media.—The media used in these experiments were as follows:

(1) McConkey's broth, double strength (Fig. 4).

(2) Lactose broth (Figs. 1 and 3).

(3) Glucose broth (Fig. 2).

(4) McConkey's agar.

Organisms.—The organisms tested were *Bact. coli* and *Proteus vulgaris* in 14- and 36-hour cultures respectively.

Antibiotics.—The antibiotics tested were aureomycin, achromycin, and penicillin, the first two in the form of powder and pellet, the last in an aqueous solution. (Aureomycin as hydrochloride, crystalline, and spersoids was obtained from the Lederle Laboratory Division ; achromycin in a crystalline powder as HCl and pellet from the same firm ; and penicillin as "procaine penicillin G" from the Commonwealth Serum Laboratories, Parkville, Victoria, Australia.)

A series of photographs was taken in some of the experiments together with the potential readings in order to compare the technique with the old method of simple observation.

Results

Fig. 1 gives the picture when *Bact. coli* is grown in the presence of increasing doses of one antibiotic, e.g., penicillin. The doses range from 0.1 μg. to 10 mg. in the following order: 0.1 μg., 10 μg., 100 μg., 1 mg., and 10 mg. in 10 ml. medium.

Fig. 3 presents the effect of a single dose (100 μg.) of each of three different antibiotics (penicillin, achromycin, aureomycin) on the same organism (*Bact. coli*).

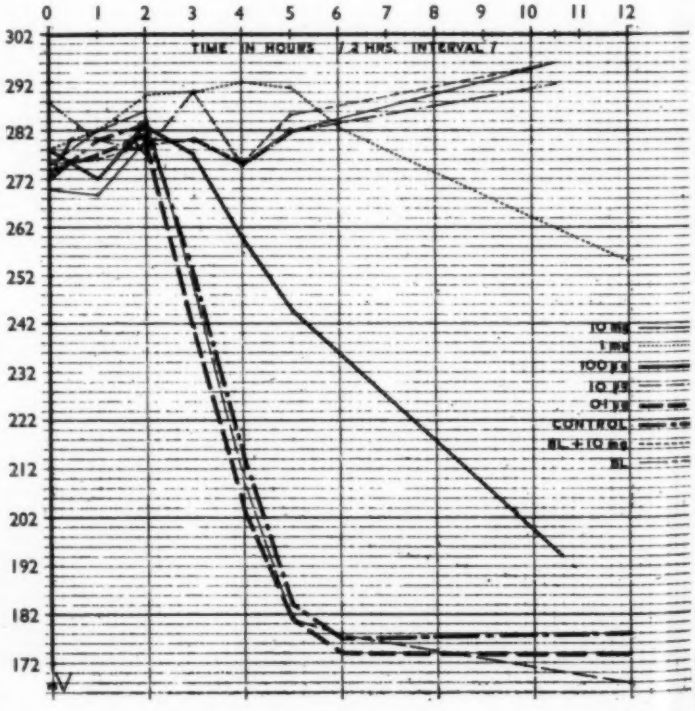

FIG. 1.—Result of growing *Bact. coli* in presence of increasing doses of penicillin.

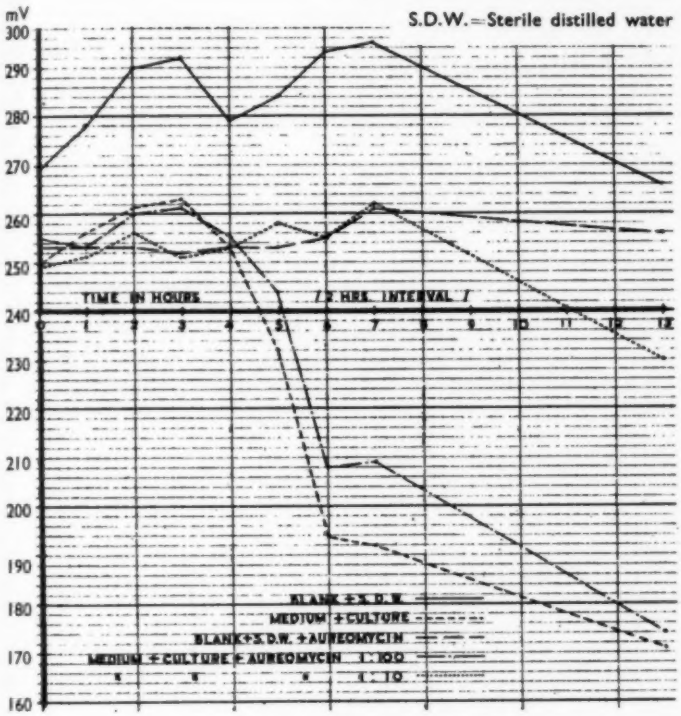

FIG. 2.—Results of growing *Proteus* in glucose broth + aureomycin.

Fig. 2, like Fig. 1, shows the effect of increasing concentrations of one antibiotic, aureomycin, 1:10 and 1:100, on the growth of *Proteus*.

Although the method is extremely sensitive, it is not influenced by such variations as height of the liquid (within limits) or metallic test-tube capsules instead of cotton-wool plugs, etc. (Fig. 4).

Discussion

Recording redox potentials in bacterial cultures is not a direct measurement, i.e., measuring the redox potentials of the bacterial organism itself, but indirect, since the potential changes of the culture medium are measured (Hewitt, 1950). When organisms are present a certain energy-producing ingredient of the medium is decomposed. The quicker the organisms grow, the more energy they need, and the greater the redox potential will be. Hence, by the close correlation between the changes in the medium and the growth of the organisms, the life of the organisms can be followed and a fairly true picture obtained by this measurement (Zador, 1958).

If there is an agent present which is likely to influence growth and metabolism in the medium in either direction (enhancing or restricting and blocking it), the chemical decomposition in the medium will alter together with the redox potential accordingly. The application of these general principles provided the basis for this technique.

The results in Fig. 1 show the differences in reaction of the organisms, although the dosage was increased only 10 times in all except one case in which it was increased 100 times.

From the experience gained from these results the technique can safely be recommended as a basis for a new method of assessing antibiotic dosage. If the other requirements, i.e., strain, age of test organism, culture medium, temperature, duration of incubation, remain the same, the amount of antibiotic can be determined with great accuracy by this method.

The method can also be used for antibiotic sensitivity tests.

On the basis of experience obtained from the results shown in Fig. 3 the sensitivity of the test will allow comparisons between the effects of the same dose of various antibiotics as applied to the same organisms under similar conditions.

These two factors, speed and accuracy, combined with simplicity and ease, make this method suitable for any type of routine work where the bacteriostatic effect of an agent has to be tested. But the great value of the test lies in the fact that it gives graphic representation of what is happening in the life of the organism. By studying these curves an exact picture of the condition or the metabolic state of the organism at any given moment can be seen, and to obtain this information it is only necessary to take readings at closer intervals or to connect the millivolt meter to an automatic recording machine.

Other photo-electric and electronic methods have been worked out, but they all fail in comparison with this method, because they gave a static rather than a dynamic picture of the metabolic processes involved.

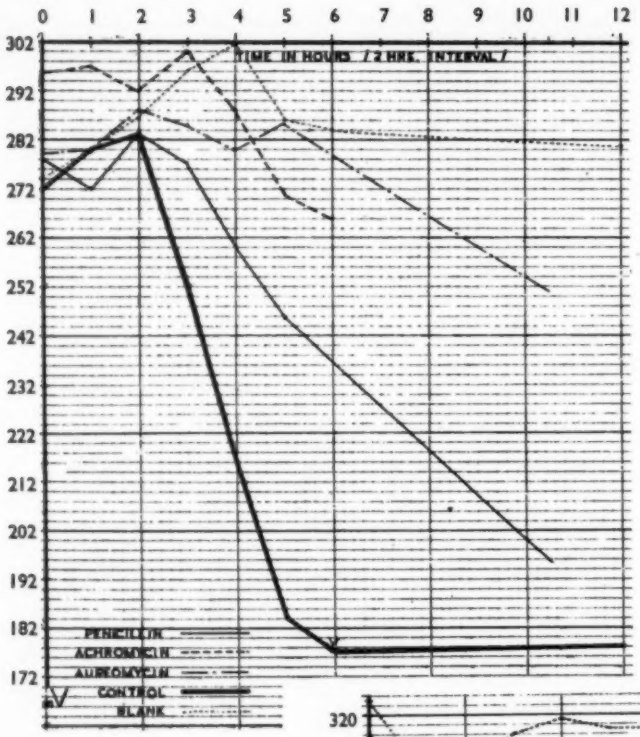

FIG. 3

FIG. 3.—The effect of a single dose (100 μg.) of each of three antibiotics on *Bact. coli*

FIG. 4.—Curves showing lack of effect of various negligible factors.

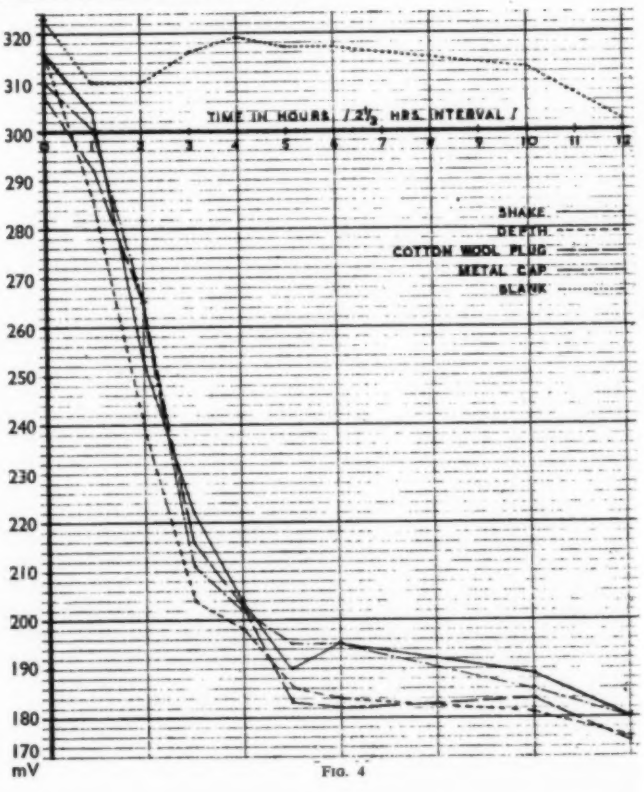

FIG. 4

Summary

There is a close relationship between the redox potential and the growth of living cells. This fact is used for developing a new method for testing the antibiotic resistance of micro-organisms by measuring redox potentials. The method is described fully, and its field of application, together with advantages and shortcomings, is discussed and the results are examined in detail.

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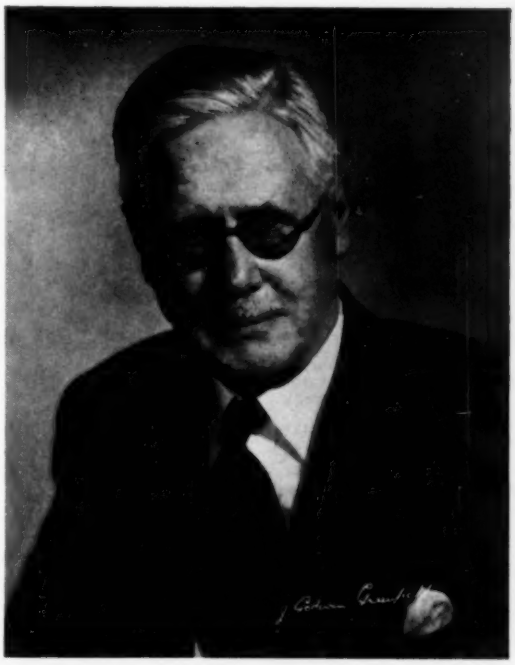
J. clin. Path. (1958), **11**, 281.

OBITUARY

J. G. GREENFIELD, M.D., B.Sc., LL.D.Edin., F.R.C.P.

Dr. J. Godwin Greenfield died suddenly, aged 73, on March 1, 1958, at Bethesda, Maryland, U.S.A., where he had spent many months each year for a number of years since he retired from the National Hospital, Queen Square, London.

He was born in Edinburgh, where his father, W. S. Greenfield, was professor of pathology and clinical medicine. There too he was educated, both at school and at the University, where he qualified in 1908. During his period of medical education he was distinguished for his prowess not only at work but also at play, for he was a University golfer, and he continued this form of exercise throughout his active life. After two years as house physician at the National Hospital, he went to Leeds General Infirmary as an assistant pathologist, where he came under the influence of Professor Matthew Stewart. He returned as pathologist to the National Hospital, but almost at once he enlisted in the R.A.M.C., and served during the 1914–18 war in Belgium and France. Later he went to Tooting Hospital, where he did valuable neuropathological work. Returning in 1919 to Queen Square to resume his appointment, he continued for the next 30 years as pathologist to that hospital, apart from a spell of work in the Emergency Medical Service at Chase Farm Hospital during World War II.



He retired officially in 1949, but for the next few years he continued with research work at Queen Square and also commenced writing the volume on neuropathology which has now been published.

Greenfield received an invitation to go to America, and for the past three years he spent part of each year at the National Institute of Neurological Diseases and Blindness at Bethesda. This proved to be a great joy to him, for not only had he considerable opportunities but every facility of that Institute was placed at his disposal, including a flat for himself and for Mrs. Greenfield, who accompanied him on each occasion.

He wrote extensively, chiefly on neurological subjects, and many are the erudite articles he contributed to journals such as *Brain*. He was co-editor with Arnold Carmichael of the well-known book on the cerebrospinal fluid, and with Farquhar Buzzard wrote a small book on neuropathology. More recently a monograph on spinocerebellar degenerations was published, and with some American colleagues a volume on muscle diseases appeared, both of which were received with well-deserved compliments by reviewers.

Many were the honours so rightly bestowed on him. Lectures he gave included the Oliver-Sharpey, the Morison, the Hughlings Jackson, and not least the second Dyke Foundation lecture of the Association of Clinical Pathologists. He was the recipient of an honorary LL.D. of Edinburgh given to him by the hand of the Duke of Edinburgh, as he then was. At this magnificent ceremony, which I was privileged to attend, he showed his typical modesty, and later his great pleasure at this honour in his home town.

Greenfield was a founder member in 1927 of the Association of Clinical Pathologists, and was immediately elected to the Council. The following year he became vice-president, in which capacity he served five years. He became President in 1934, holding that office for three years. He then returned to the Council for four years, and in 1948 he was appointed to the Editorial Board of the *Journal of Clinical*

Pathology, on which board he remained for nine years, retiring only one year ago. Throughout this long period Greenfield worked quietly but continuously to further the work of the Association, for, although the foremost neuropathologist in this country, yet he was a typical example of a real clinical pathologist. His knowledge of general pathology was very wide and he was an extremely able general histologist, as I know from personal experience. He gave of his knowledge and wisdom not only to the Association but also to the Consulting Pathologists Group of the British Medical Association.

Towards the end of his life he founded the Neuropathological Club in an endeavour to aid the many younger members who were interested in this subject, and this club included overseas as well as British members. This thought was typical of his interest in others and for the welfare and encouragement of the more youthful pathologist.

I first met Greenfield in 1933 when he was an acknowledged master, yet his modesty, sincerity, and kindness towards his new and young assistant pathologist was something that will never be forgotten. Further, his manner of teaching, his methods of guidance and direction, were not lost upon the many with whom he came in contact. Always enthusiastic, always helpful, he would assist in whatever was necessary, even to the cutting of his own histological sections, for in this too he was an expert. The very many postgraduate students who came to work under him were impressed not only by his knowledge but also by the inherent friendliness of a very human man, and many have continued in correspondence with him throughout the years. Housemen and postgraduate students were invited to play tennis with him at the weekends, and at Christmas there was always the exchange of gifts. In later years, after he moved to Surrey, many friends of all age groups visited the Greenfields and were entertained by them. He enjoyed showing them round his garden, for there he spent much time working hard and finding great pleasure in the fruits of his labours. He was, as well as being a gardener, a keen photographer, and, while colour transparencies were his favourites, his Christmas cards of recent years have usually been decorated by a black and white landscape taken by him during the year.

Despite osteoarthritis of the hip he continued to enjoy life to the full and it appeared as though he would be working for many more years.

It is with much sorrow that we lament his passing, but we are very grateful to have known and worked with him. It will be extremely difficult to fill the gap caused by his death either in the Association or in neuropathology.

JOHN N. CUMINGS.

ALFRED S. GIORDANO

Alfred S. Giordano was elected to honorary membership of the Association of Clinical Pathologists in 1930 when the total membership numbered but 82. At that time he was unknown to us except as the secretary of our counterpart society in the United States which had come into existence four years before ours and which by then was already well established.

Alfred Giordano was born in Avellino, Italy, in 1893, and at the age of 4 was taken to Syracuse, New York, where he went to high school and subsequently to the university, qualifying there in 1920. After

spending three years in postgraduate study at the Mayo Clinic he was appointed pathologist to the South Bend Medical Laboratory, a diagnostic centre established in 1912 by a group of far-sighted practitioners and housed at that time in the Epworth (now the Memorial) Hospital. In this pleasant industrial town of some 115,000 inhabitants, he spent his whole professional life, and at the time of his retirement in 1952 this unit had grown under his able direction from a single room in a basement manned by one technician and himself to a well-equipped institute and associated hospital laboratories with a medical staff of seven and a technical staff of 75. In 1946 the central laboratory became the South Bend Medical Institution, a non-profit-making diagnostic and research organization, and now a permanent memorial of his vision and enterprise.

And here it was in this model laboratory that Jerry Giordano made everyone feel welcome. After a visit to the Foundation the traveller from abroad would be taken to his lovely home set in a wooded garden sloping steeply down to a picturesque bend on the St. Joseph river. After a serious illness in 1948 he was compelled to abandon this delectable home in favour of a single-storied house on level ground, but here too

was a lovely garden and here too he continued his interest in music. Since 1952 he and his wife wintered in Sarasota, Florida, and it was there that he died in his sleep on February 15, 1958.

Giordano has been proclaimed as having probably done more for clinical pathology than any other person (*Amer. J. clin. Path.*, 1949, **19**, 51). During his 17 years' secretaryship of the A.S.C.P., unfortunately terminated by his illness, he saw many changes, and it was during this time, very largely due to his efforts, that clinical pathology in the United States became recognized as a specialty and its practitioners afforded the place in the profession which they deserved. He was partly instrumental in founding the American Board of Pathology and, in order that the Society might be free to pursue its scientific and educational programmes unhampered by medico-political matters, the College of American Pathologists.

As an individual Jerry was charming. Although he disliked the limelight he enjoyed innumerable friendships, was a delightful host and he excelled in a small company. It is a thousand pities that his illness prevented him from coming to England to attend, as he so much wanted to, one of our meetings: he valued greatly his honorary membership of our society and would have felt very much at home with us. He worked all his life to improve the standing of hospital pathologists and especially the men in those communities which have no university at hand. It was for this reason that the American Society of Clinical Pathology, partly at his instigation, embarked on an educational programme designed primarily to help the lone pathologist. "The pathologist," wrote Giordano to the writer of this notice, "is the spiritual scientific leader of his medical community." He therefore deprecated in America the employment of pathologists by hospitals on a whole-time basis because it discouraged the younger men of the better type from entering the field. Pathologists did not want to be hired men, he said. "The financial rewards of the pathologist," he wrote, "should be in proportion to their activity and interest." Only in this way did he believe the right types would be attracted into the specialty.

Alfred Giordano's was a life of service for others, for his fellow townsmen in South Bend, and for his fellow pathologists and the profession generally the world over. We extend our sympathy to his wife, Dr. Alice Giordano, to his two doctor sons, one of whom is a pathologist, and to his two daughters. We should like them to know that we in the United Kingdom appreciate all that he has, by his example, done for us, and we are grateful.

W. H. McMENEMEY.

The papers on hospital coccal infections which formed a symposium arranged by the Association of Clinical Pathologists and the Medical Research Council Committee on Cross Infection in Hospital have been edited by R. E. O. Williams and R. A. Shooter. The booklet (price 3s. 6d.) may be obtained from the Swan Press Ltd., Eakers Row, Farringdon Road, London, E.C.1.

J. clin. Path. (1958), **11**, 284.

BOOK REVIEWS

General Pathology, 2nd ed. Edited by Sir Howard Florey. (Pp. xv + 932, illustrated. 84s.) London: Lloyd-Luke. 1958.

It has been a long-standing tradition in medical education in this country that a proper appreciation of systematic pathology can only be acquired after a sound grounding in the principles of general pathology, and that these in turn should be closely grafted upon those of physiology. It arose with Roy and Sherrington, pupils of the Cohnheim rather than the Virchow school, and it has since been fostered by the series of well-known textbooks by Adami, Lazarus Barlow, and Pembrey and Ritchie. At the present time, in spite of the growing transatlantic emphasis on organic pathology, it is still fortunately strong, and no better evidence of its vitality in our time could be found than in this Oxford book. In 43 chapters, Florey and his collaborators have covered the whole field of general pathology; many in the present edition are revised versions of those that formed the original "Lectures," but several are wholly new.

In the first edition a notable omission, which drew comments from many reviewers, was the absence of any treatment of neoplasms. This deficiency has now been made good by four substantial chapters by Berenblum, in which he takes up successively the nature of their growth, their classification and morphology, their epidemiology, and their experimental production in animals. As is to be expected, all four are admirable surveys of present knowledge and provide the student with a comprehensive review of the problems of tumour pathology which he will meet with increasing frequency while he passes through his clinical years. A further valuable addition is a chapter on atherosclerosis by J. E. French, in which, beginning with the recognized morphological features of the typical lesions, he passes on to an excellent review of the recent studies on the associated biochemical changes in the blood and tissues, many of which are now being actively prosecuted at Oxford. A welcome feature of these new chapters is the perspective given to the reader by relevant vital statistics—the product of a branch of medicine that has been more carefully cultivated in this country than anywhere abroad. How rapidly the relative importance of the different diseases, measured in terms of morbidity and mortality, is changing can only be appreciated through frequent references to the reports of the Registrar-General and to other sources of epidemiological material. The long process of eclipse, at first gradual but recently dramatic, of the formerly common infectious diseases and their replacement as causes of death by neoplasms and vascular diseases make these new chapters by Berenblum and French particularly opportune.

Other new chapters which enhance the value of the book are those by Pickering on fever, now again a subject of lively study, by French on thrombosis, and by Born on the effects of injury on metabolism. This last discussion is especially noteworthy, for the applications of modern endocrinology and biochemistry to the problems raised by trauma and by the cachexia of neoplasia may well lead shortly to advances in surgery which will much extend its useful applications to treatment in both conditions.

In a book which reaches so uniformly high a standard of presentation, it would seem invidious to criticize any single contribution. But it would appear to the reviewer that, when this book reaches its next edition, greater attention could, with advantage, be given to the diseases caused by viruses and to the reactions of cells to their presence. Not only are these diseases becoming of increasing importance, but the relations of these infective agents to the host cells in which they multiply are raising issues of great interest in both general biology and pathology.

In his preface, Florey points out that the book is intended for the "better student" in the hope of arousing his interest in the functional as well as the morphological aspects of pathology. For this, there could be no more stimulating book. The presentation throughout is on scholarly lines, the statements generally supported by those references to original literature that proper education now requires, and the whole superbly illustrated by a very large number of diagrams and beautiful half-tone photographs. The book is a tribute to the present Oxford school of pathology, and a reminder of the great potentialities of the experimental approach when it is applied to this science. There can be no doubt that it will appeal to, and influence the outlook of, many students, and will further strengthen the more distinctively English belief in the intimate dependence of pathology on functional as well as on morphological studies.

G. PAYLING WRIGHT.

Analytical Pathology, 1st ed. Edited by R. C. Mellors. (Pp. 550; 105 figures. £5 16s. 6d.) London: McGraw-Hill. 1957.

This is a collection of essays on certain aspects of eight topics of importance to pathologists: cancer, arteriosclerosis, inflammatory and vascular disease of the kidney, liver failure, the anterior pituitary, macrocytic anaemia, abnormal haemoglobins, and hypersensitivity in relation to connective tissue diseases.

The stated aim in each case is a review of some aspect or aspects of the chosen topic in the perspective

of recent advances in biology, chemistry, or physics which have illuminated the topic. Inevitably, in a work of this kind, under multiple authorship, the extent to which this aim is realized varies considerably. There is similar variation in the matter and manner of presentation of the material discussed.

For example, the contribution on cancer by the editor of this collection surveys nearly 600 papers on clinical and experimental aspects of carcinogenesis in about 100 pages. This compresses a remarkable amount of factual information into the space available and yet even finds room to discuss briefly the speculative and all-embracing concepts of the genesis of cancer arising from the work of Weiss and of Green.

The article by Gofman on arteriosclerosis and hypertension, on the other hand, devotes 50 pages to the narrow limits of discussing changes in the blood lipids, demonstrable by the ultracentrifugal techniques developed by this author and his collaborators, in relation to statistical considerations of the incidence in coronary arteriosclerosis. No new evidence is presented to show a causal relationship between alterations in serum lipoproteins and coronary disease and there is a somewhat polemic discussion of alternate hypotheses such as that of Duguid.

The articles by the pathologists among the contributors, i.e., on the kidney by D. B. Jones, on the anterior pituitary by Agnes Russfield, and on connective tissues by R. M. Wagner, describe the application of the newer staining techniques and histochemical methods to the investigation of disease processes affecting these tissues and attempt the correlation of altered cellular morphology with biochemical changes demonstrated by other methods.

The remaining articles are by physicians. The discussion by Ratnoff on three aspects of hepatic failure, cholaemia, ascites, and haemorrhagic phenomena, is selective rather than exhaustive but excellent in its treatment of modern work in this field. The attempt by Mueller and Vilter to formulate the pathogenesis of the various varieties of macrocytic anaemia in terms of blockage of various metabolic pathways for nucleoprotein synthesis is stimulating though as yet premature in the light of deficiencies in present knowledge in this field. The essay on abnormal haemoglobins by Will adds little to the numerous reviews already available on this topic and is already outdated by the discovery of further abnormal haemoglobins and by other recent work on the pathogenesis of hereditary haemolytic anaemias.

Most of the articles are well-documented, many listing between one and two hundred references each. While these are, as usual, largely to American sources, British work is not overlooked. Although the price of this volume is somewhat high it will serve as a useful reference source to those actively engaged in research on the topics discussed.

KENNETH WALTON.

Histological Technique, 3rd ed. By H. M. Carleton, with the collaboration of R. A. B. Drury. (Pp. xvi+343; 19 figures. 30s.) London: Oxford University Press. 1957.

The appearance of the third edition of "Carleton," 19 years after the second edition, constitutes a fitting memorial to this distinguished Oxford histologist, who died late in 1956, just after completing the manuscript. In preparing this edition, the author had the benefit of collaboration with a pathologist, Dr. R. A. B. Drury, and a short foreword has been added by Professor Sir G. Roy Cameron.

Many pathologists—and even more pathological technicians—will have had a nostalgic attachment to the old "Carleton," which was for so many of us the bible which guided our first attempts at histological technique. For many years now, however, our old editions have rested more and more on the shelf as it has been necessary to use other sources for the newer techniques—both empirical and histochemical—which are gradually adding to, or replacing, the older methods. It is natural that we should look carefully at this new edition to see if the revisions effected can re-establish the volume's overall usefulness in the pathological laboratory.

We find that the volume ranges over an enormous field. It deals not only with tissue preparation, sectioning and staining, but also with the theory and practice of microscopy including dark-ground illumination, fluorescence microscopy, and even electron microscopy. When one adds that there are chapters on injection methods, histochemical techniques, methods for a great variety of special tissues, cells, cell structures, bacteria, and parasites, and that the volume remains a relatively small and convenient one, it is perhaps redundant to point out that the treatment of many of these subjects is superficial. For example, the periodic-acid-Schiff technique (not indexed in its own right) is given barely half a page for a description of the procedure and its significance; and the important methods for tissue enzymes, such as the phosphatases and esterases, are completely omitted.

But these are carping criticisms, for no volume of this size and price could have met the requirements of the advanced worker as well as the novice. This new revision brings "Carleton" back to its place as an eminently suitable guide for the technician up to his final examination in histological technique and for the pathologist in his earlier exploits amongst routine methods.

T. CRAWFORD.

Handbook of Histopathological Technique (Including Museum Technique). By C. F. A. Culling. (Pp. x+446+index; 79 figures. 45s.) London: Butterworth. 1957.

New technical methods are constantly being evolved, and Mr. Culling's book contains detailed accounts not only of the older and already stan-

dardized methods but also of many others developed during the past 10 years. It is divided into an introductory section on the cell and methods of examining cells (nine pp.) and further sections on fixation, processing, and section cutting (70 pp.), stains, impregnations, and mountants (160 pp.), special procedures, including autoradiography and microincineration (11 pp.), museum technique (27 pp.), and the microscope (55 pp.). Each section includes an appropriate amount of information on the theories underlying the techniques recommended.

A wide range of staining methods is described, almost always simply and adequately, and often with added details, small in themselves but important in practice, that clearly reflect the author's own experience in the laboratory. Several useful tables are included, amongst them a stain solubility chart, buffer tables, and schemes for identification of lipid and P.A.S.-positive materials. The section on the microscope includes descriptions of the compound, dark-ground, fluorescence, polarizing, phase-contrast, and interference machines, and even a short note on the electron microscope. Though 55 pages long, this section makes available in one place much information that has otherwise to be sought in several. The chapters on museum technique will be useful to workers in many laboratories. The manufacture of perspex specimen jars is not difficult (though finding time may be!): the clear instructions here, combined with a visit to some laboratory already making jars, should bring it within the range of any competent technician.

In a text almost innocent of misprints (there is one on p. 292, second last line) it is surprising to find so many errors in proper names: Perl's for Perls's (throughout, including the frontispiece), Grubler for Grübler (throughout), Heiffor for Heiffer (pp. 89–91, though correct in index), McNanus for McManus (in bibliography, though correct in text), Hutchinson for Hutchison (in index and bibliography, though correct in text), and a misrendering of Dr. A. G. E. Pearse's name in the bibliography. In figure 31 the legends for posterior horn and posterior root have been transposed.

A few further points might merit attention in a second edition. The corrosive effects of mercuric chloride on metal (p. 27) can be resisted by containers of "monel" metal available for use in automatic tissue processing machines. Gelatin embedding may be useful for uterine curettings (p. 79), but there should rarely be difficulty with ordinary paraffin techniques. The section on knife sharpening (pp. 95–97) could usefully include some mention of the flat disc or turntable type of sharpener so widely and successfully used in the U.S.A. The statement (p. 217) that fixation is not critical for the demonstration of fibrin does not apply to the picro-Mallory methods: the best results follow two to three weeks' fixation in mercuric chloride. Enough has by now been discovered about mast cells to make obsolete the statement (p. 294) that "the function of these cells is not known": and it is an over-simplification to say that the epidermis consists of two layers (p. 332). Perfect sectioning of the eye is sufficiently difficult to warrant greater technical detail than there is on pp. 335–6: equatorial bisection, as recommended here, is usually a less valuable preliminary cut than two slices that "take off the top and bottom," leaving the whole of the cornea, lens, and optic nerve in one flat circular block.

In general, however, this is a book that well deserves a place on the shelves of any histopathological laboratory. Indeed, a laboratory would do well to have two copies, one for reference, the other for borrowing, for the author has had in mind the needs of candidates for the final examination of the I.M.L.T. It has clear type, a pleasing format, and a waterproof, acid-resistant binding.

W. W. PARK.

The Biologic Basis of Cancer Management. By Freddy Homburger. (Pp. xviii+354; 39 tables, 10 figures. 75s.) London: Cassell. 1958.

The title of this book is promising. So is its dedication, "To Gin," and so also are the forewords, three of them, by a pathologist, a research worker, and a surgeon.

In the preface which follows, the author explains how the book came to be written; in reply, it seems, to questions put to him as a "cancer co-ordinator." He is disarmingly modest about the wisdom of writing it: "the idea of the biologic basis of cancer management was indeed rejected by a publisher because of the opinion of eminent consultants that no one person could write such a book." Unfortunately they were right.

In the first place the title is very misleading. Thus 126 pages are devoted to the aetiology of cancer compared with four to the principles of surgery. The principles of radiotherapy receive 10 pages but contain only a scant reference to the variable radiosensitivity of tissues and their derived tumours. McWhirter's treatment of breast carcinoma is mentioned but not discussed, nor is any estimate attempted of the true value of early surgery or of periodic check-ups in the detection of early cancer.

By deleting the word "management" perspective is restored but many weaknesses remain. Over-condensation has led to superficiality and in places also to imbalance. Two pages on the relation of smoking to lung cancer contain no relevant figures and no mention of Wynder and Graham or Doll and Bradford Hill. This enables Dr. Homburger to remain securely on the fence—perhaps he is a heavy smoker. Elsewhere we have sentences such as: "It appears from the rate of cancer incidence that about 20% of the population are susceptible." To what? to the present level of environmental carcinogens? and in what life-span? The sentence is either a tautology or untrue, since, as the author points out further on, if we live long enough 80% of us will develop cancer of the prostate.

In addition there are far too many errors; "megakaryocytes" for "megaloblasts" on p. 141 is a glaring example, and table 8 is full of them.

In fairness it must be said that the second section on "the biologic behaviour of cancer" contains some interesting material, this being Dr. Homburger's own field of study. But the relative merits of the middle do not redeem the whole book.

HUMPHREY KAY.

Comparative Aspects of Haemolytic Disease of the Newborn. By G. Fulton Roberts. (Pp. xi+199. 17s. 6d.) London: William Heinemann Medical Books. 1957.

For his first chapter Dr. Fulton Roberts has unearthed some interesting historical facts about haemolytic disease of the newborn. As early as the seventeenth century the occurrence of more than one case of neonatal jaundice in a family had been noted. In the middle of the nineteenth century a veterinarian, in describing some cases of neonatal icterus in newborn foals, was put in mind of a neighbour who had lost several successive infants from jaundice.

The association of anaemia with severe neonatal jaundice was noted by Auden in 1905, and earlier (1892) Ballantyne described the association of anaemia with hydrops foetalis. Recognition that icterus gravis and hydrops were linked was possibly delayed by the fact that one form or the other tends to recur in the same family, so that a woman may have a dozen badly jaundiced infants in a row without one hydropic infant. However, between 1922 and 1931 several authors concluded that there was a definite disease entity which might be manifested by jaundice or oedema. The ground was thus prepared for Diamond, Blackfan, and Baty's well-known review, published in 1932.

The rest of the book is devoted to a detailed survey of the published work on haemolytic disease of the newborn in man and animals. There are a few statements to which exception may be taken: it is not the "oxygen carying power of each red cell" which "is suddenly improved at birth" (page 36) but the oxygen saturation. It is a little misleading to say (pages 43 and 44) "How vitamin K promotes the hyperbilirubinaemia in man is not clear" since in Allison's first reported cases signs of haemolytic anaemia were noted. The two-component curve of elimination of incompatible cells which has been described in man can hardly be due to exhaustion of complement (page 134) since it is observed when less than 1 ml. of cells is injected. These and a few similar passages do not seriously detract from the value of the book, which will be found a most useful source of references to anyone interested in haemolytic disease.

P. L. MOLLISON.

Burns: Pathology and Therapeutic Applications. By Simon Sevitt. (Pp. xii+364; 85 figures. 37s. 6d.) London: Butterworth. 1957.

This small, convenient, well-written, and well-produced volume brings together almost everything that is known concerning "the pathology and disordered physiology after burning." The physical aspects of burning, the histopathology of the lesions, including healing and disturbances of healing and the bacteriology, epidemiology, and control of infection, are dealt with in detail. Chapters follow dealing fully with mortality and causes of death, the physiopathology of burn shock, other metabolic effects, and the effects in each of the systems of the body. Finally there are accounts of the special features of flash, radiation, electrical, and chemical burns, and full author and subject indices.

Throughout the book there is the fullest consideration of therapeutic relationships and full documentation of all the aspects discussed. It would be difficult to imagine a more complete and lucid account of a limited subject. The book provides an unparalleled source for references on its subject and must prove invaluable for all—clinicians as well as pathologists—who are regularly concerned with the handling of these distressing injuries.

T. CRAWFORD.

Fluid Balance in Surgical Practice, 2nd ed. By L. P. Le Quesne. (Pp. vii+140; 42 figures. 20s.) London: Lloyd-Luke. 1957.

In presenting the second edition of this popular book, the author has revised the text to correct a disproportion between the sections in the original work and to incorporate recent advances. The monograph includes an account of the physiology of the body fluids and the metabolic response to surgery, with sections on water, salt, and potassium depletion and brief chapters on renal failure, water and salt excesses, nutritional problems, and fluid balance in children. An appendix giving a number of illustrative case histories is a useful feature.

This is indeed a wide field to be covered in 140 pages, but the author has successfully achieved this by selecting a practical approach with emphasis on the recognition and management of disturbances in fluid and electrolyte balance.

It is considered that acid base upsets have received undue attention in the past, and it is perhaps unfortunate that little space has been devoted to their consideration. The reader seeking information on this topic will therefore be disappointed. One might also have expected some mention of the use of plasma substitutes such as dextran in patients with severe circulatory collapse. As a routine basic fluid intake post-operatively, the generous figure of 3,000 ml. per 24 hours is recommended, a dose that would appear in some cases to carry a risk of water intoxication.

Though primarily intended for the practising surgeon, who cannot afford to be ignorant of the principles and considerable practical information contained therein, this book includes much that will interest and enlighten the laboratory worker.

H. W. C. AULD.

Essentials of Chemical Pathology. By D. N. Baron. (Pp. xii+247; 28 figures. 25s.) London: The English Universities Press. 1957.

Dr. Baron, in 200 pages, summarizes with remarkable clarity the application of biochemistry to the day-to-day problems of chemical pathology. The text is supplemented by some excellent tables. I like particularly a separate table which gives the normal values for children.

This book could be read with pleasure and reward by senior registrars as well as by senior students. It is well indexed, totally devoid of frills, and many busy clinical pathologists will find it a valuable, though inexpensive, addition to the library of reference books.

Unfortunately there are a certain number of somewhat irritating type-setting errors, suggesting hasty preparation. These, with a few textual errors which are so obvious that they will not mislead, can easily be corrected at the next reprinting.

N. H. MARTIN.

The Nature of Brucellosis. By Wesley W. Spink. (Pp. xiv+464; 56 figures. 63s.) Minnesota University Press (London: Oxford New Press). 1957.

The publisher states that "in this volume, Dr. Spink, a world authority on the disease, presents a comprehensive review of human and animal brucellosis and the results of important new research." This is certainly true, as there is scarcely a phase of this fascinating disease that Dr. Spink has not adequately covered. The opening chapter gives an accurate review of the evolution of the concept that brucellosis is a disease of animals and man, the early work of British, American, and Maltese scientists being fully described. The chapters on epidemiology and pathogenesis are quite outstanding. The description of the clinical features of the disease is admirable. Appendices comprise over 100 pages and include the protocol of 244 cases of brucellosis. The book is to be recommended to anyone anxious to gain accurate information on brucellosis, and it should be invaluable to the clinical pathologist. The style is always interesting, and the publishers have produced a manual which is a pleasure to read.

J. V. WILSON.

BOOKS RECEIVED IN 1957

(Review in a later issue is not precluded by notice here of books received.)

Die arterio-venösen Anastomosen. By Max Clara. (Pp. vii+315; 101 figures. £5 4s.) Vienna: Springer-Verlag. 1956.

Hématose spontanée, plaquettes sanguines et parois vasculaires. Ier Symposium de la Fondation Valentine Baldacci (Madrid, 27–30 mai, 1955). (Pp. 190; 5 figures.) Pisa: Edizione "Omnia Medica." 1956.

Physiopathology of the Reticulo-Endothelial System. A Symposium organized by the Council for International Organizations of Medical Sciences. (Pp. xii+317; illustrated. 45s.) Oxford: Blackwell Scientific Publications. 1957.

Blood and Bone Marrow Patterns. By G. D. Talbott, Elmer R. Hunsicker, and Jonah Li. (Pp. 59; 145 colour plates. $12.) New York and London: Grune and Stratton. 1957.

Les Nouveaux Syndromes Hémorragiques: La Dysprothrombie. By Paul Chevallier and A. Fiehrer. (Pp. 128; 25 figures. Fr. fr. 1,500.) Paris: Masson. 1957.

La Puberté: Étude Clinique et Physiopathologique, 2nd ed. By Guy Laroche and collaborators. (Pp. 394; 42 figures. Fr. fr. 2,800.) Paris: Masson. 1956.

An Introduction to Blood Group Serology: Theory, Techniques, Practical Applications, Apparatus. By Kathleen E. Boorman and Barbara E. Dodd. (Pp. viii+317; 30 figures. 40s.) London: J. and A. Churchill. 1957.

Radiologie Clinique de L'Estomac Opéré. By P. Porcher and P. Buffard, in collaboration with R. Guillet and Cl. Couinaud. (Pp. 660; 507 figures. Fr. fr. 7,200.) Paris: Masson. 1957.

Maladies des Veines. By Claude Olivier; Foreword by Prof. H. Mondor. (Pp. 600; 342 figures. Fr. fr. 6,700.) Paris: Masson. 1957.

Transactions of the American Goiter Association 1956. (Pp. xiii+473; illustrated. £6 6s.) Oxford: Blackwell Scientific Publications; Springfield, Illinois: Charles C. Thomas. 1957.

The Clinical Application of Antibiotics, Vol. III: Chloramphenicol and the Tetracyclines. (Pp. ix+393; illustrated. 84s.) London: Oxford University Press. 1957.

Cerebral Lipidoses: A Symposium. *Chairman:* L. van Bogaert; *Editor:* J. N. Cumings; *Associate Editor:* A. Lowenthal. (Pp. x+212; illustrated. 42s.) Oxford: Blackwell Scientific Publications. 1957.

Clinical Pathology Data, 2nd ed. By C. J. Dickinson; Foreword by C. E. Dent. (Pp. xiii+91. 20s.) Oxford: Blackwell Scientific Publications. 1957.

The Clinical Aspects of Arteriosclerosis. By Seymour H. Rinzler. (Pp. xiii+339; 57 figures, 11 colour plates. 66s.) Oxford: Blackwell Scientific Publications; Springfield, Illinois: Charles C. Thomas. 1957.

Mechanisms of Hypertension: With a Consideration of Atherosclerosis. By Henry Alfred Schroeder. (Pp. xv+379; 39 figures. 67s. 6d.) Oxford: Blackwell Scientific Publications; Springfield, Illinois: Charles C. Thomas. 1957.

Spondylitis Andylopoetica (Der Rheumatismus, Vol. 3). By Victor R. Ott and Hans Wurm. (Pp. xii+246; 137 figures. DM.38.) Darmstadt: Verlag Dr. Dietrich Steinkopff. 1957.

J. clin. Path. (1958), **11**, 101.

A STUDY OF SURGICALLY REMOVED SPECIMENS OF BREAST, WITH SPECIAL REFERENCE TO SCLEROSING ADENOSIS

BY

A. T. SANDISON

From the Departments of Pathology, the University and Western Infirmary, Glasgow

(RECEIVED FOR PUBLICATION JULY 13, 1957)

It is now generally accepted that carcinoma of the breast is not restricted to the middle-aged or elderly woman and that a mass in the breast in a woman of any age must be regarded as malignant until it is proved to be benign. This recognition, which has recently been re-emphasized elsewhere (Sandison, 1955), has led to a considerable increase in the demand by surgeons for immediate examination of frozen sections in the theatre in cases of a mass in the breast of younger women. In many instances such examinations will quickly show whether or not a lesion is malignant. The appearance known as sclerosing or fibrosing adenosis may present, especially to the less experienced pathologist, a puzzling picture, and one which may erroneously be diagnosed as carcinoma. In sections prepared by the paraffin embedding method the appearance is less likely to be misinterpreted as carcinoma, but even here difficulty in interpretation has been known. This difficulty is especially important, since sclerosing adenosis is most characteristically seen in younger women in whom mastectomy leads to greater mental distress than in older women. It therefore seems worth while to indicate the frequency with which this picture of sclerosing adenosis has been encountered in the examination of surgically removed breasts or portions of breasts in a five-year period in this laboratory during which just over 1,000 breast specimens were received.

The salient features of sclerosing adenosis may be outlined as follows. It produces a firm, mobile nodular or discoid lump in the breast, most frequently in younger women, and is thought to be due possibly to some temporary hormonal imbalance (Dawson, 1954). The naked-eye appearance is that of a greyish-white nodular mass with hyperplastic lobular areas sometimes associated with cysts. Histological examination confirms the lobular nature of the lesion—a most important diagnostic point. The cytological character varies with the phase at which the condition is seen. There is an early florid phase (Fig. 1) which regresses and goes on to an actively sclerosing stage and a final fibrosed lesion (Fig. 2). In the florid phase epithelial and myoepithelial hyperplasia in the lobules dominates the picture with the formation of narrow tubules or solid cords of cells. The stroma is delicate, the cells active, and mitotic figures may be present (Figs. 3–5). Later, as the lesion regresses, there is proliferation of connective tissue stroma till finally the epithelial cords are attenuated and compressed by fairly dense hyaline connective tissue. This appearance is one readily mistaken for scirrhous spheroidal cell carcinoma of the breast, especially if viewed only under the high power of the microscope. It cannot be too strongly emphasized that in the study of breast tissue, especially in frozen sections, very great weight must be given to the impression obtained under very low magnification with a scanning objective (e.g., × 4) and preferably a wide-angle eye-piece, which gives a well-illuminated large field.

Method

In all instances the original reports of the naked-eye and microscopic findings were consulted and the sections were re-examined, but the cases were allocated to groups according to the diagnosis made on re-examination of the sections. These groups consisted of:

(*a*) True neoplasm, i.e., carcinoma, fibroadenoma, and papilloma.

(*b*) The mastopathies subdivided into hyperplastic cystic disease, characterized by intraductal or intra-acinar hyperplasia (epitheliosis), and simple fibrocystic disease in which there was no epithelial hyperplasia but which might show some degree of lobular hyperplasia (adenosis), and to this group sclerosing adenosis is probably related.

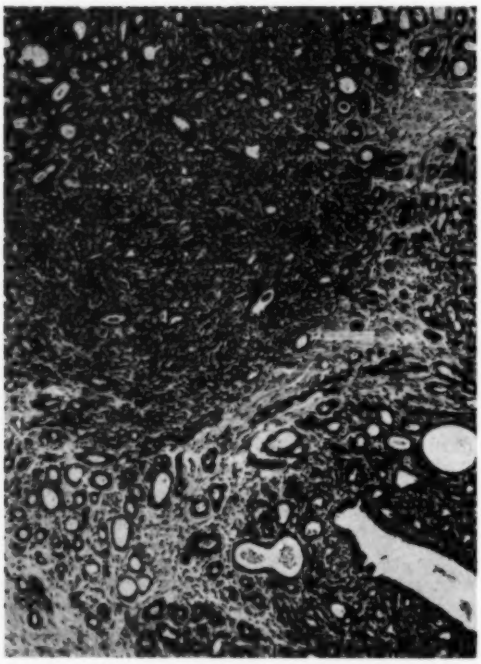


FIG. 1.—Low-power view of sclerosing adenosis (florid phase) in a portion of the right breast of a 27-year-old woman who complained of a lump in the breast for three weeks. Haemalum and eosin, ×65.

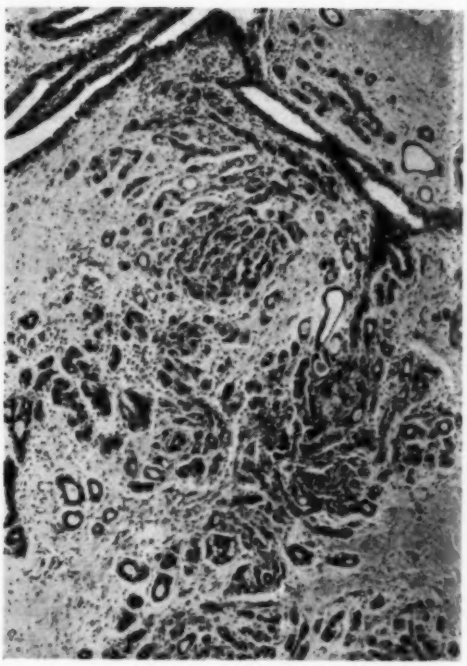


FIG. 2.—Low-power view of sclerosing adenosis (sclerosed phase) in portion of right breast of a 40-year-old woman who complained of a lump in the breast after pregnancy two years previously. Haemalum and eosin, ×65.

(*c*) The infective mastitides, lipophagic reaction, lactation effects, and a small miscellaneous group.

Sclerosing adenosis was also regarded as a primary group where it appeared probable that it was the condition for which surgery had been undertaken. A further note was made also of all cases in which sclerosing adenosis was an incidental finding. In this way the minimal incidence has been established. The occurrence of sclerosing adenosis as a secondary feature in breast pathology is much higher than as a primary condition presenting as a palpable mass. It is therefore possible that frozen sections of breast in other conditions may show foci of sclerosing adenosis, the recognition of which is important to avoid mistakes in diagnosis.

Material

The survey comprised a review of the sections of all breast or portions of breast received in this department over a four-year period from 1950 to 1954. The total number of specimens examined was 1,029, of which 1,010 were female.

The major findings in the male breasts are classified in Table I, and those in the female breasts in Table II. Sclerosing adenosis does not occur in the male because of the absence of lobular formation.

Included in the miscellaneous group were two lipomata, one large simple adenoma, one epithelial tumour of low-grade malignancy, one angio-endothelioma, one secondary melanoma, two cases of tuberculosis, eight mammary duct ectasias, and one simple mammary hypertrophy.

The age distribution of the lesions is shown in Table III, from which some facts of interest emerge. It will be seen that fewer specimens were received from women in the second decade of life than in any other until the ninth decade. Under the age of 20 fibro-adenoma was the common lesion while the only other seen was intraduct papilloma. Two of the fibro-adenomata were of foetal type. In the third decade occasional cases of carcinoma appeared, although fibro-adenoma was much the commonest lesion, with occasional cases of infective mastitis and lactation effect. In

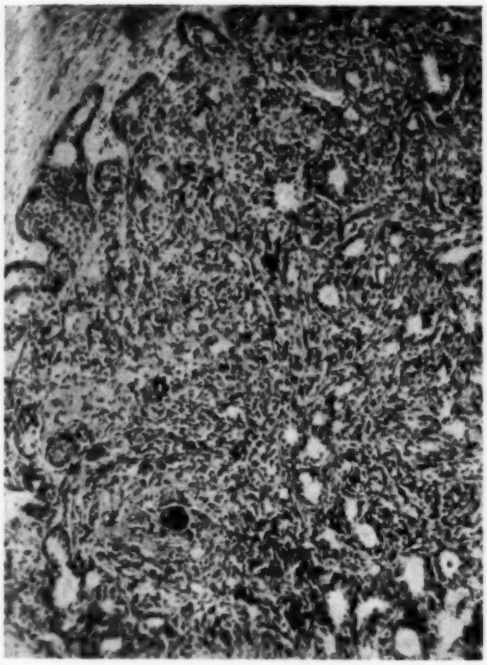


FIG. 3.—Sclerosing adenosis in the florid phase in the left breast of a 48-year-old woman who complained of a lump in the breast for one year. Haemalum and eosin, ×90.

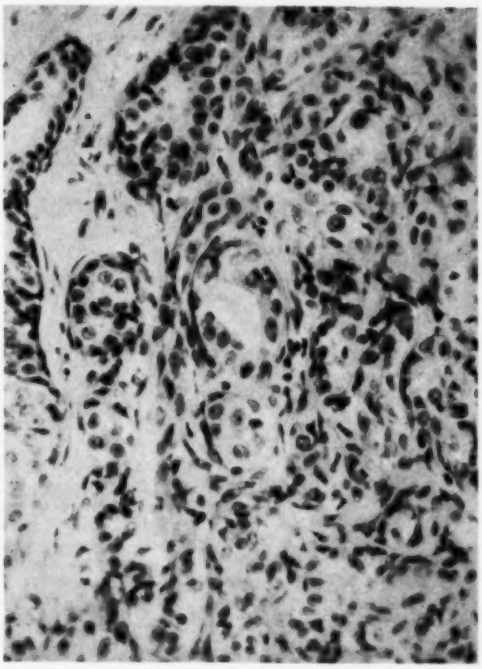


FIG. 4.—Higher power view of florid phase sclerosing adenosis in the same case as Fig. 3 to show great cellularity. Haemalum and eosin, ×250.

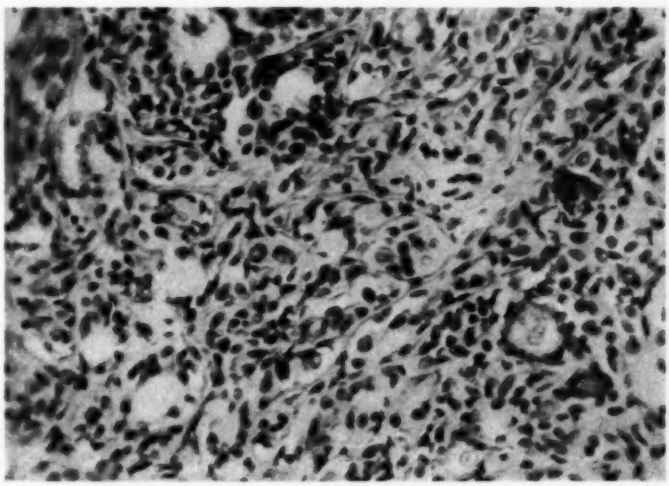


FIG. 5.—Another field from the same case. The myoid element is well shown. Haemalum and eosin, ×250.

TABLE I
FINDINGS IN MALE BREASTS

Diagnosis	Number of Cases
Carcinoma	3
Physiological hypertrophy or gynaecomastia	15
Infective mastitis	1
Total	19

TABLE II
FINDINGS IN FEMALE BREASTS

Diagnosis	Number of Cases
Carcinoma	524
Fibro-adenoma	110
Giant fibro-adenoma	6
Papilloma of ducts	37
Hyperplastic cystic disease	98
Simple fibrocystic disease	155
Pure sclerosing adenosis	12
Infective mastitis	27
Lipophagic reaction	11
Lactation effect	4
Miscellaneous	26
	1,010

the fourth decade carcinoma was as frequent as fibro-adenoma. Infection and lactation effects were still seen, but now hyperplastic cystic disease (epitheliosis) was fairly common. In the fifth decade the maximum incidence of breast specimens was attained. Carcinoma was by far the most frequent lesion with hyperplastic cystic disease and fibrocystic disease each much less common. Fibro-adenoma was now relatively infrequent, but papillomata were at their maximum incidence. Occasional cases of infection still occurred. In the sixth decade the frequency of carcinoma was unchanged, but hyperplastic cystic disease, simple fibrocystic disease, and fibro-adenoma were relatively rare. Infective mastitis still occurred in the seventh decade; the frequency of carcinoma was again relatively unchanged, but all other conditions were relatively rare. In the eighth decade the number of cases of carcinoma was only half of that seen in each of the previous three decades, while over the age of 80 carcinoma, although uncommon, was the sole lesion for which surgery was attempted.

Of 524 carcinomas, 40 occurred in women under the age of 40, while in the next three decades the incidence remained remarkably stable at about 130, falling off to about half that number in the eighth decade. Simple fibrocystic disease, hyperplastic cystic disease, and duct papillomata lead most frequently to operation in the fifth decade. It may be that these are in fact most common at about the time of the menopause or that in this period of life surgery is more frequently advised.

Fibro-adenoma occurred from the second to the eighth decade but is commonest in the third and fourth. Giant fibro-adenoma was only about one-eighteenth as common and occurred most frequently in the seventh decade. Duct papillomata were twice noted in young girls, but were seen most often in the fifth decade. Infective mastitis was largely a disease of the third and fourth decades, i.e., the reproductive period, while fat necrosis occurred in the same group and also rarely affected older women.

Of the male breasts examined all three carcinomas were seen after the age of 50, but of the cases of gynaecomastia or hyperplasia almost as

TABLE III
AGE DISTRIBUTION OF MALE AND FEMALE CASES

Age	Females												Males			
	Carcinoma	Fibro-adenoma	Giant Fibro-adenoma	Duct Papilloma	Hyperplastic Cystic Disease	Simple Fibro-cystic Disease	Pure Sclero-sing Adenosis	Infective Mastitis	Lipophagic Reaction	Lactation Effect	Miscellaneous	Total	Carcinoma	Physiological Hypertrophy (Gynaecomastia)	Infective Mastitis	Total
10–19	—	9	—	2	—	—	—	—	—	—	—	11	—	3	—	3
20–29	2	37	—	1	5	10	4	9	—	2	2	72	—	1	—	1
30–39	38	34	1	4	24	33	3	8	3	2	7	157	—	3	—	3
40–49	139	18	—	15	54	81	5	3	2	—	5	322	—	1	—	1
50–59	135	6	—	7	6	15	—	5	4	—	5	183	1	1	1	3
60–69	123	—	3	3	3	9	—	1	—	—	5	147	1	2	—	3
70–79	63	2	1	3	1	2	—	—	—	—	2	74	—	4	—	4
80–89	9	—	—	—	—	—	—	—	—	—	—	9	1	—	—	1
90–99	1	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—
Age uncertain	14	4	1	2	5	5	—	1	2	—	—	34	—	—	—	—
Total	524	110	6	37	98	155	12	27	11	4	26	1,010	3	15	1	19

many were seen before this age as after it. These included three instances in boys in the second decade, which are best regarded as adolescent hyperplasias.

Sclerosing Adenosis

Sclerosing adenosis was present in breast specimens from 12 women as a relatively pure primary lesion. The age distribution may be seen in Table III. In three of those women in whom mastectomy had been done because of an associated papillomatosis with nipple discharge, sclerosing adenosis was present both in the original biopsy and in the breast when removed. In the remaining nine women biopsies were done in four instances and primary local mastectomies in the remaining five.

Of these women, 10 were married and only two single, and the history of a lump varied from three weeks to 13 years. In one unmarried woman aged 44 the lump had been present for 13 years and was said to have followed a non-puerperal abscess. In another, a married woman aged 39 years, the lump was thought to have followed a puerperal abscess eight years before. In the majority of cases there was no history of gross menstrual upset. One 24-year-old woman had, however, noted a mass during the fifth month of her only pregnancy two years previously; the child was not breast-fed, and for seven months before operation there had been severe menorrhagia. Another woman, aged 44, also complained of menorrhagia for three months before operation. In one instance a woman aged 29 had had, three weeks before mammary biopsy, an abdominal operation at which the pelvic organs were seen to be entirely normal.

In the majority of instances the clinical impression was one of mobile, firm nodularity of breast substance although occasionally a discoid mass was felt. In only one woman, aged 48, had the nipple discharged; the discharge was purulent in appearance and when examined cytologically (Sandison and Walker, in preparation) contained histiocytes or "colostrum corpuscles," some red blood cells, and epithelial cells. This appearance is characteristic of papillomatosis and was due, not to the sclerosing adenosis, but to a concurrent multiradiculate papillomatosis. It may be of importance to note that this woman had had, shortly before mastectomy, a course of oestrin for menopausal symptoms.

Microscopically all of the cases showed a lobular hyperplasia with myoepithelial and epithelial participation. In six cases, of which two occurred in the second decade, one in the third, and three in

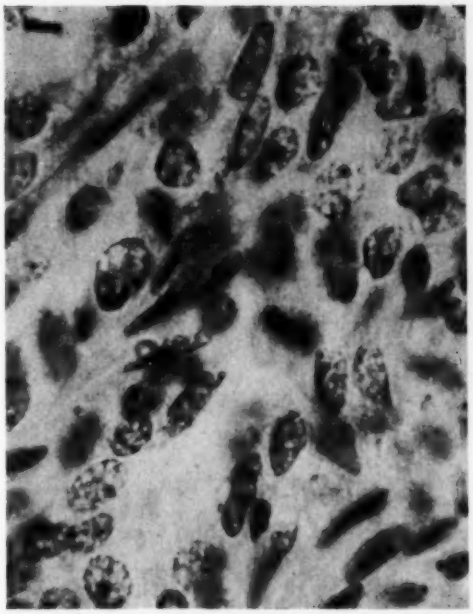


FIG. 6.—Mitosis in florid sclerosing adenosis in a biopsy from the right breast of a 31-year-old woman who had complained of a lump for five years, becoming larger more recently. Haemalum and eosin, ×1,000.

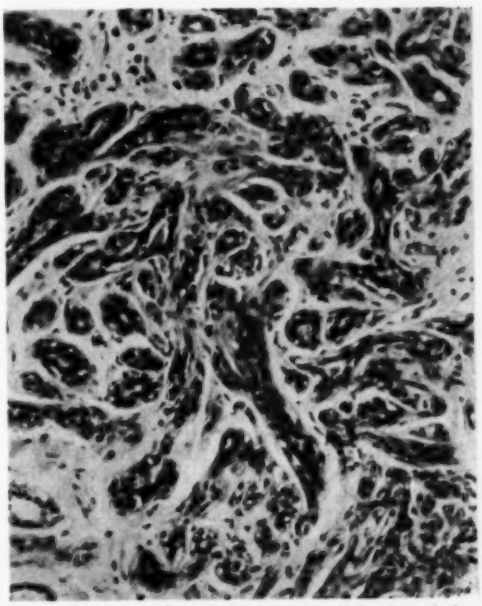


FIG. 7.—High-power view of sclerosing adenosis in sclerosed phase showing cords of involuting cells which may be mistaken for scirrhous carcinoma. Haemalum and eosin, ×160.

FIG. 8.—Rosette of lobules around a terminal duct; all of these show sclerosing adenosis. Haemalum and eosin, ×10.

the fourth, the appearances were largely of the florid type. In the instance occurring in the third decade mitotic activity was especially marked (Fig. 6). In the remaining cases the florid phase was less obvious, more or less fibrosis had occurred, and the appearances tended to mimic those of scirrhous infiltrative carcinoma (Figs. 2, 7). In 10 cases there was some degree of microcystic formation and in seven there were also cysts lined by metaplastic "pink" epithelium of so-called apocrine type. In only two instances was there associated epitheliosis of ducts, but in nine there were minute papillomata in ducts. This association appears to be significant.

In three cases where both biopsy and later mastectomy specimens were available the condition was seen to be present in both. In cases where primary mastectomy was carried out, the condition, although most obvious in the focal lesion for which the operation was done, was also present to a similar or lesser extent in other regions of the breast.

In one woman, who was aged 44 and unmarried, there was a peculiar rosetted arrangement around a central fibrous core (Fig. 8). This is considered to be a change in several lobules arranged at the termination of a single duct.

Sclerosing adenosis as a complicating lesion was present as a secondary lesion in 73 breasts; 28 of these were carcinomatous, six contained large papillomata, and two fibro-adenoma, 23 were associated with only simple fibrocystic disease, 13 with hyperplastic cystic disease, and one with infective mastitis. The age distribution of these cases is shown in Table IV with that of primary sclerosing adenosis.

TABLE IV

SCLEROSING ADENOSIS AS COMPLICATING LESION

Age	Primary Sclerosing Adenosis	Carcinoma	Papilloma	Fibro-adenoma	Simple Fibrocystic Disease	Hyperplastic Cystic Disease	Infective Mastitis	Total
20–29 ..	4	—	1	—	1	—	1	7
30–39 ..	3	1	1	—	5	5	—	15
40–49 ..	5	10	4	2	11	5	—	37
50–59 ..	—	10	—	—	3	2	—	15
60–69 ..	—	3	—	—	2	1	—	6
70–79 ..	—	4	—	—	1	—	—	5
Total	12	28	6	2	23	13	1	85

Of these 28 women with carcinoma, six were single and 22 were married. One woman, aged 49, had undergone a course of oestrin therapy for menopausal symptoms shortly before mastectomy. Another, aged 50, had a further mastectomy about 18 months later and the second breast also showed well-marked sclerosing adenosis as the primary condition. In this case the condition was therefore bilateral. Yet another woman, aged 64, died several days after mastectomy, and it was possible to examine the remaining breast at necropsy. This showed epitheliosis, papillomatosis, cyst formation, and sclerosing adenosis in both the florid and fibrosing stages. In the breast removed for carcinoma the condition was largely in the more florid stage. The ovaries at necropsy showed a fairly cellular stroma according to the criteria of Sommers and Teloh (1952) and the endometrium was rather hyperplastic. This case is of especial interest because the appearance of the endometrium suggested some degree of continuing oestrogen activity. In two instances it was of interest to note areas of incomplete involution of lactation effect, in view of the belief that perversion of lactation may play a part in the aetiology of the lesion.

In some instances it may be difficult to differentiate between sclerosing adenosis and scirrhous carcinoma when both are present in the

same section. The lobular distribution of the changes in sclerosing adenosis provided a useful basis for differentiation, and detailed comparative cytological examination may reveal considerable differences. It is, in fact, a salutary exercise to attempt this differentiation in cases where the two types of lesion are closely intermingled. In one instance, in a woman aged 48, a carcinoma of largely intraductal and intra-acinar type was complicated by the presence of sclerosing adenosis of rather florid degree and in which the component elements were difficult to distinguish. This is not surprising, since both intra-acinar carcinoma and the proliferative phase of adenosis are essentially lobular, highly cellular, and both may show active growth with mitotic figures. It is also interesting that in this case a part of the area of active adenosis showed some degree of apocrine metaplasia.

Six women showed sclerosing adenosis as a secondary lesion to papillomatosis of the breast. This association of papillomatosis and sclerosing adenosis is of considerable interest since it will be recalled that in nine women who were considered to show sclerosing adenosis as the primary breast condition there were also associated microscopic papillomatosis. In a total of 85 women who showed either primary or incidental sclerosing adenosis in some degree, some 15 also showed papillomatosis in some degree, i.e., just over 17%. On the other hand, of 37 women who showed papillomatosis as a primary condition only, six showed sclerosing adenosis, a proportion of 16%. Of these six women showing an association of sclerosing adenosis with primary papillomatosis, only one was unmarried. One of the women, aged 25, complained of menstrual irregularity. Of two women who showed sclerosing adenosis in association with fibro-adenoma, both were married, in the fifth decade of life, and no history of menstrual irregularity was obtained. One fibro-adenoma was intracanalicular and the other of mixed type.

Simple fibrocystic disease of the breast was the commonest primary condition to be associated with sclerosing adenosis. Twenty-four cases in all were noted. Of these, five women were known to be unmarried. In none of these cases was epitheliosis or intraduct hyperplasia a feature. In the majority there was some degree of apocrine metaplasia in the cysts and also frequently a varying degree of simple adenosis of physiological type. One breast from a 35-year-old woman showed evidence of incomplete involution of lactation.

Sclerosing adenosis complicated hyperplastic cystic disease, in which there was some degree of epitheliosis or intraduct hyperplasia, in 13 cases (13%). Of these women three were unmarried. In only one instance was there a definite history of menorrhagia. Three of the cases showed quite marked epitheliosis; in the remainder the hyperplasia was of only moderate degree.

The one instance of sclerosing adenosis as a secondary feature in chronic infection occurred in a 27-year-old married woman who showed a non-specific chronic infection of six years' duration. In this instance only one small focus of sclerosed adenosis was seen.

When all cases showing sclerosing adenosis in some degree are considered together the incidence was 28 instances in 524 cancerous breasts (5.3%) and 57 instances in 486 non-cancerous breasts (12.2%).

Discussion

Sclerosing adenosis although recognized was not named by the older authorities, e.g., Muir (1941) or Cheatle and Cutler (1931). A brief description of this condition is given by Ewing (1940), who points out that more than any other condition in the breast it is likely to be mistaken for carcinoma. Since then this warning has been reiterated by Boyd (1947), while Foot (1945) mentions "diffuse fibrosing adenomatosis" but contents himself with stating that its presence does not alter the prognosis of fibrocystic disease. Saner (1950) regards the condition as a form of hyperplastic cystic disease which may give rise to difficulty in diagnosis on immediate examination in the theatre.

Mulligan (1951) gives a useful summary of his views on sclerosing adenosis. It is much more commonly seen as an incidental microscopic finding than as a presenting discrete mass which is in any case rarely large. The lesion is most commonly found between the ages of 20 and 40 years; it begins within the lobules or terminal ductules and in the early stage there is considerable proliferative activity. Throughout the evolution of the lesion a distinct lobular pattern persists. In the later phase connective tissue tends to compress irregularly the attenuated cords of epithelial cells and a bizarre pattern may thus be produced distinctly reminiscent of scirrhous carcinoma.

Willis (1953) also emphasizes this collapse, atrophy, and distortion of the epithelial elements with stromal fibrosis, and states that there is a superficial resemblance to infiltrating scirrhous carcinoma. He states that mistaken diagnoses may be made, especially in frozen sections, and that the only safeguard is experience in the recognition of fibrosing adenosis at the different evolutionary stages and insistence on paraffin sections to confirm all frozen section diagnoses.

Ackerman (1953) has also summarized his views in an addendum to his remarks on chronic cystic mastopathy. He considers sclerosing adenosis to be a highly proliferative form of simple cystic disease of rather uncommon type, and gives the incidence as 1% of benign breast lesions.

Stewart (1950) gives a detailed consideration of fibrosing adenosis in the section on lesions simulating carcinoma in his atlas of breast tumours. Stewart points out that the fibrosing phase is that most commonly recognized, but that it is also important to recognize the florid phase of epithelial proliferation. The age incidence given is similar to the experience of Mulligan (1951). Stewart also emphasizes that this lesion is especially liable to be over-diagnosed by pathologists who only encounter small numbers of breast specimens. He describes instances where not only mastectomy but post-operative radiotherapy and castration had been recommended, and deprecates the unnecessary "single-breasted" state of not a few young women in the third decade of life because of this diagnostic failure.

He gives detailed accounts of the morbid anatomy and histology of the lesion, which is discrete, often coarsely nodular, less firm than carcinoma, and usually distinctly lobulated. Stewart concedes that sclerosing adenosis may occur also in microscopic foci and that the only real difference between this form and that detected as a palpable mass is that the former occurs in miniature.

Ingleby and Gershon-Cohen (1954) have recently reviewed the problem of adenosis generally. They state that in the normal breast in the post-menstrual phase ductules bud to form new lobules with deeply staining cells. As the cycle progresses both epithelial and basal cells become paler and larger and myo-epithelial cells are formed by formation of blunt processes. Some few days before menstruation begins some secretion appears in the ducts, while the epithelial and basal cells vacuolate. During the period the basal cells break up and the epithelium is shed into the lumen. In the premenstrual phase also the intraductal stroma is loose and oedematous, with scattered mononuclear cells, while after the period this stroma is dense and fibrous.

Ingleby and Gershon-Cohen classify adenosis into four types. Type A consists of lobular hyperplasia with very large lobules or numbers of smaller lobules close together, while in type B, which is the commonest form, the lobules consist of a few more or less dilated ductules with incomplete lobular development. They state that type C occurs near the tumour in most carcinomas of young women, that epithelial and basal proliferation may be noted, and that duct epitheliosis may also be present. Type D is sclerosing adenosis, and Ingleby and Gershon-Cohen think this is often formed entirely of myo-epithelial cells and therefore prefer to entitle this lesion "myoid sclerosis." The validity of this concept of sclerosing adenosis as well as that of possible precancerous potentiality in their type C adenosis remains uncertain.

Valuable papers on sclerosing adenosis have come from Urban and Adair (1949) and Foote and Stewart (1945). The latter authors noted that the lesion was present in some degree in 12.5% of 200 non-cancerous and 7% of 300 cancerous breasts studied, and these figures do not differ greatly from mine, i.e., 12.2% and 5.3%.

Dawson (1954) has published a comprehensive descriptive article on fibrosing adenosis emphasizing the fact that it is often unrecognized. She points out not only the possibility of overdiagnosis and the subjection of the patient to unnecessary operative risk, but also of vitiation of statistical figures for cancer survival rates. She points out that in itself the term "adenosis" carries no pathological implication, and that, especially in older women, involution after pregnancy without lactation may be slow. Studies of post-mortem breasts in this department, to be published later, confirm this observation (Sandison, 1958).

Post-menopausal adenosis may be due to continued hormone secretion by the adrenal cortex or from other sites. Novak (1952) has speculated on a similar basis for the noted occurrence of post-menopausal endometrial hyperplasia seen in women without functional ovarian tumours. It is of interest to note at this point that Geschickter (1945) illustrates a case of mastodynia in a patient who received over one million i.u. of oestradiol benzoate over a period of 20 months, and the photomicrograph shows apparent sclerosing adenosis.

Dawson emphasizes that recognition of the phases of normal growth with possible irregularity or perversion is necessary for an understanding of fibrosing adenosis. She has also found fibrosing adenosis to be commonest in younger married women and frequently associated with some perversion of mammary function. It does, however, occur in younger unmarried subjects and in older patients. Fibrosing adenosis as a localized tumour-like condition she has found to be rare. It is, however, not uncommon as a focal change in the breast as seen in surgically removed specimens or at necropsy.

With regard to histological studies, Dawson, who publishes a large number of photographs, agrees that it is essential to recognize the pattern of growth. Dawson's description of the microscopic appearances corresponds closely with those of Mulligan (1951) and Stewart (1950), and with all of these the present study also corresponds closely. Dawson rightly points out that staining by silver methods or by phosphotungstic-acid-haematoxylin shows an intact basement membrane round the distorted glands or cell groups with no evidence of invasion, and that mitoses may occur in epithelial cells in the florid phase and in fibroblasts in the actively sclerosing phase. Dawson suggests that epitheliosis is largely absent in the ducts in sclerosing adenosis, but with this statement the present study is not in full agreement. Epitheliosis may be present although it is often mild. Dawson concedes that epitheliosis may be seen in association with sclerosing adenosis in older women and thinks that the two are not related. It seems possible, however, that disordered hormonal balance may lead to papillary epitheliosis as well as adenosis and the two may not be mutually exclusive. Since it is generally believed that epitheliosis of duct sometimes precedes the development of intraduct neoplasia, the occasional association of sclerosing adenosis and carcinoma is not surprising. Nevertheless it should be emphasized that there is no causal relationship between sclerosing adenosis and mammary neoplasia. It is generally accepted that the sclerosing phase represents an involutionary process which becomes to all intents and purposes quiescent.

There do not appear to be any reports concerning the incidence of sclerosing adenosis in breasts examined at necropsy, but in this department a complete study of the breasts in 800 consecutive female necropsies (Sandison) showed uncomplicated sclerosing adenosis in 3.1% of all cases, the staging of the lesions being 36% in the florid, 36% in the sclerosing, and 28% in the sclerosed phase. A further 3.9% of all cases showed sclerosing adenosis as well as other changes. A detailed report of the findings in this study will be published in due course.

Summary

The minimal incidence of sclerosing adenosis in surgical specimens of breast, both as a primary condition and complicating other lesions, has been established.

In just over 1,000 female breasts there were 12 primary and 73 secondary instances of the condition. The result of a simple analysis of the total number of specimens examined is also presented.

The significance of sclerosing adenosis is discussed, and the importance of its recognition by the practising surgical pathologist is emphasized.

I am grateful to Messrs. G. Kerr and W. Mason for the photographs illustrating the paper.

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THE STAINING REACTIONS OF CHORDOMA

BY

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Although chordoma is a rare tumour well over 300 cases have been reported, and the excellent published descriptions of both pathological and clinical features leave little to be added (Stewart, 1922; Cappell, 1928; Mabrey, 1935; Dahlin and MacCarty, 1952). The pathological diagnosis depends on the recognition of the physaliphorous or "bubble-bearing" cells which are the most striking feature of the histological picture. These cells (Fig. 1) often attain great size and usually occur in groups or cords with an epithelial pattern; they are distinguished by the presence of numerous clear vacuoles of varying size which are separated by faintly eosinophil cytoplasm, the cell having almost an aerated appearance. The groups of cells are separated by a matrix or ground substance which has a similar frothy vacuolated appearance to the cell cytoplasm. In some areas —mainly the older parts of the tumour—the cells collapse to nondescript stellate and fusiform varieties and are often separated quite widely by this frothy ground substance (Fig. 2). Biopsy from such an area presents considerable difficulty and has often led to an erroneous diagnosis of chondrosarcoma, especially when the tumour occurs in one of the less common sites such as a spinal vertebral body. In these circumstances some staining reaction which would distinguish between the ground substance of cartilage (more particularly neoplastic cartilage) and chordoma would be invaluable. The present paper presents from this point of view a study of material from six cases of chordoma.

Material and Methods

Some details of the six cases from which the pathological material was obtained are given in Table I. These cases presented the usual features of patients with chordoma, though Case 1 came into the rare group in which blood-borne metastases develop, while in Case 2 the tumour involved the upper dorsal vertebrae, which are the least frequently affected parts of the spine—indeed Chiasserini and Marchiafava (1956) were able to find reports of only 10 cases with this localization.

TABLE I
CLINICAL SUMMARIES OF CASES STUDIED

Case	Age	Sex	Site	Treatment	Metastases	Follow-up
1	42	M	Sacral	Operation + radiotherapy	Lung	Died 12 years from onset and 21 months after operation
2	57	F	Dorsal	Operation + radiotherapy	—	Well 6 years after operation
3	29	M	Clivus	Operation	—	Died 10 days after operation
4	55	F	Sacral	Operation + radiotherapy	—	Recurrence treated x rays. Alive at 3 years
5	37	F	Clivus	Operation	—	Well 3½ years
6	68	M	Sacral	Biopsy only	—	Died of urinary infection 6 years after onset

Material from all the cases was fixed in formol-saline and embedded in paraffin, and in addition frozen sections were cut from formalin-fixed blocks from Cases 1, 3, and 4 for sudan staining. The staining methods employed were Ehrlich's haematoxylin and eosin, van Gieson's method, Mallory's phosphotungstic-acid-haematoxylin, the picro-Mallory method (Lendrum and McFarlane, 1940), the reticulin stain of Gordon and Sweets (1936), Best's carmine, and Southgate's mucicarmine. For the special histochemical procedures, including the methylene blue extinction test, the methods recommended by Pearse (1953) were followed. For the methylene blue extinction tests a veronal-acetate buffer was used and the *p*H of each tube was checked with a glass electrode *p*H meter.

The chance occurrence of pulmonary metastases in Case 1 enabled a direct comparison, in the same sections, between the reactions of normal bronchial cartilage and the chordomatous metastases (Fig. 3). A parallel series of preparations from a block showing pulmonary metastases of a chondrosarcoma of femur allowed the comparison to be extended to neoplastic cartilage.

Results

The cytoplasm of cartilage and chordoma cells frequently contains glycogen, giving a positive reaction with Best's carmine and staining strongly with the periodic-acid-Schiff (P.A.S.) technique. These reactions are dispelled by pre-treatment of the sections with diastase, but remain after treatment with hyaluronidase.

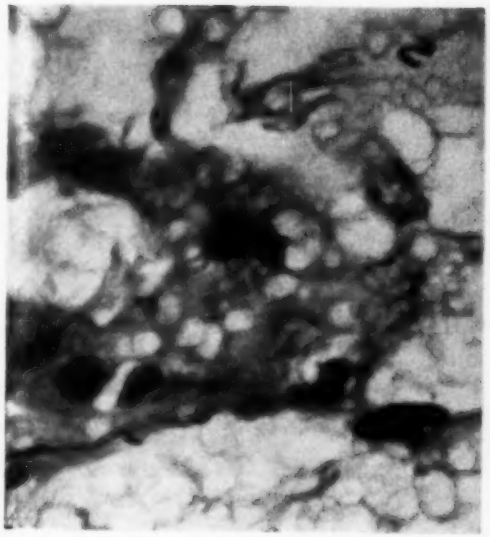


FIG. 1

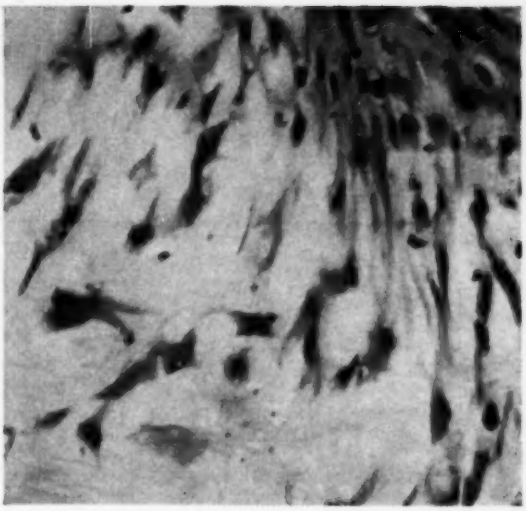


FIG. 2

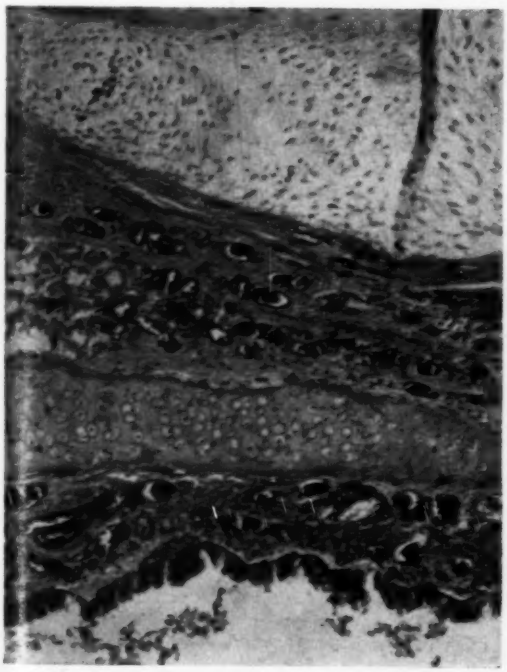
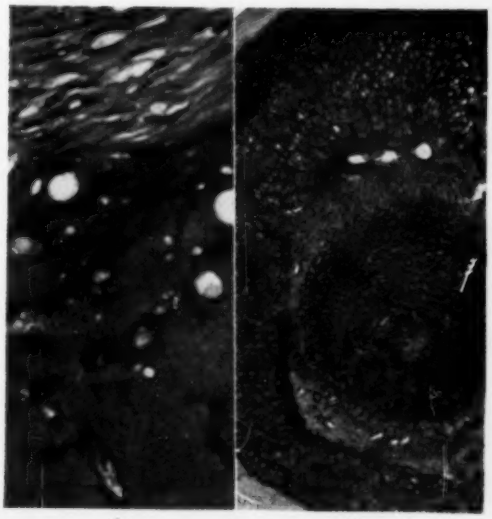


FIG. 3



(*a*) (*b*)

FIG. 4

FIG. 1.—Physaliphorous cells and vacuolated matrix. P.A.S., ×500.

FIG. 2.—Stellate and fusiform cells in abundant pale-staining ground substance. P.A.S., ×250.

FIG. 3.—Section of lung from Case 1 enabling direct comparison between staining reactions of bronchial cartilage below and chordomatous metastasis above. Haematoxylin and eosin, ×45.

FIG. 4.—Metachromatic reaction with toluidine blue. (*a*) Chordomatous metastasis, ×200; (*b*) chondro-sarcomatous metastasis with neoplastic cartilage, ×50.

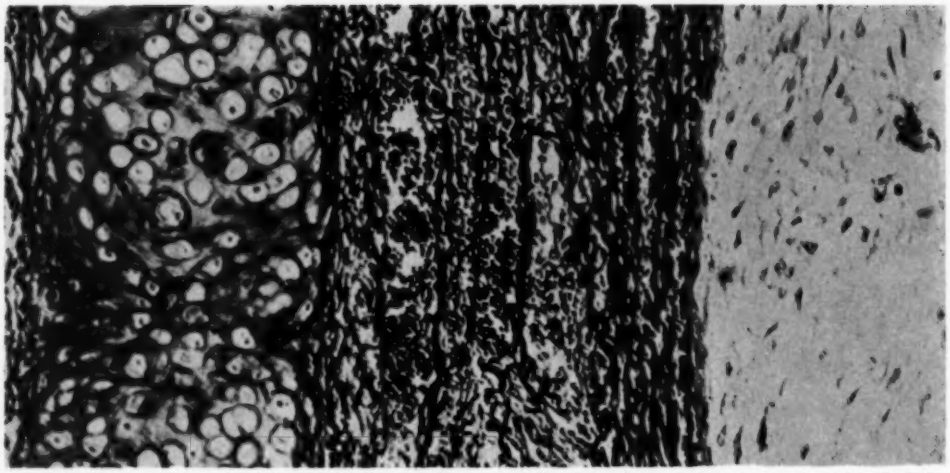


FIG. 5.—Section of lung from Case 1 showing bronchial cartilage to the left and chordomatous metastasis to the right. Reticulin stain, ×150.

The staining reactions of the ground substances of the two tissues are summarized in Table II. The positive Millon reaction given by both tissues merely indicates the presence of tyrosine-containing proteins. The P.A.S. staining given by both tissues (though usually stronger with cartilage than with chordoma) is of the type associated with mucins of connective tissue origin, rather than the more brilliant staining of the mucins of epithelial origin. The character of this staining is unchanged when the sections are previously treated with diastase or hyaluronidase. Chordomatous ground substance gives a more strongly metachromatic reaction with toluidine blue than does normal bronchial cartilage, but the neoplastic cartilage also gives a strong reaction (Fig. 4). Both tissues give similar positive staining with mucicarmine and with alcian blue. The methylene blue extinction test, with both tissues, gives a value between *p*H 3 and *p*H 2.

Turning to the more orthodox and empirical staining procedures listed in the lower part of

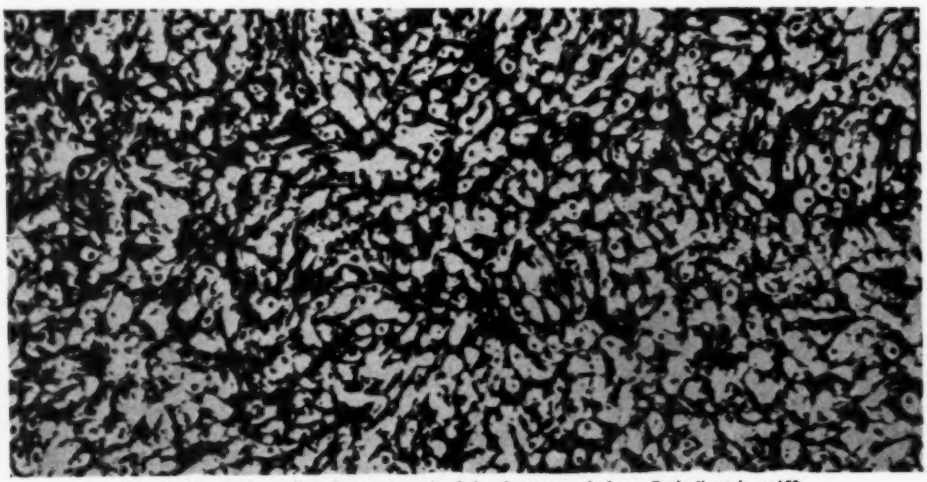


FIG. 6.—Neoplastic cartilage from metastasis of chondrosarcoma in lung. Reticulin stain, ×150.

TABLE II
STAINING REACTIONS OF GROUND SUBSTANCE OF CARTILAGE AND CHORDOMA TISSUE

Method	Chordoma	Cartilage
Millon	Positive	Positive
P.A.S.	Faint pink	Pink
,, after diastase	Unchanged	Unchanged
,, ,, hyaluronidase	,,	,,
Toluidine blue	Metachromatic	Metachromatic
Mucicarmine	Positive	Positive
Alcian blue	,,	,,
Methylene blue extinction	*p*H 2·3–2	*p*H 2·3–2
Sudan III	Negative	Negative
Haematoxylin and eosin	Almost unstained	Variable
van Gieson	,, ,,	Pink
Picro-Mallory	Blue	Deep blue
Phosphotungstic-acid-haematoxylin	Unstained	Brownish pink
Reticulin (Gordon and Sweets)	,,	Diffuse staining

Table II, it is interesting to find that these provide a better differentiation between the two substances than do the supposedly more precise methods above. With all these empirical techniques chordomatous matrix stains feebly or not at all whereas the cartilaginous ground substance gives reactions approaching more nearly those of collagen fibres. Perhaps the most clear-cut distinction is obtained with the reticulin stain. This gives quite heavy deposition in both normal and neoplastic cartilage, sharply outlining the cells and precipitating more diffusely in the intervening ground substance. The chordomatous tissue, on the other hand, remains completely unstained (Figs. 5 and 6).

Discussion

The histochemical reactions recorded lead to the conclusion that the reacting substances in both cartilage and chordoma fall into the category of acid-mucopolysaccharide—a conclusion in agreement with that arrived at by Iurato and Leonardelli (1956) from study of three examples of rhinopharyngeal chordoma. It is of particular interest that these two substances, produced as they are by tissues of such widely different embryological origin and separated so widely in their phylogenetic history, should resemble one another so closely in their histochemical reactions. The significance of this observation is obscure, but it might perhaps provoke speculation on the influence of function on structure even at the chemical level.

From the diagnostic point of view, these histochemical reactions are of little assistance. The diagnosis of chordoma depends essentially on the recognition of physaliphorous cells lying in a mucinous matrix—features which may be more distinctive in a P.A.S.- or toluidine-blue-stained section than in a routine haematoxylin-and-eosin preparation. The absence of staining of the ground substance with phosphotungstic-acid-haematoxylin and with the reticulin method may, however, provide a useful pointer when typical physaliphorous cells are absent from the section.

Summary

Material from six cases of chordoma, one of which had pulmonary metastases, has been studied. The staining reactions have been compared with those of normal and neoplastic cartilage. The mucin stains give the reactions of acid-mucopolysaccharide with both cartilaginous and chordomatous ground substance, and the two tissues cannot be clearly distinguished by these means. The sharpest histological distinction is achieved by the phosphotungstic-acid-haematoxylin and the reticulin stains. With both these techniques chordomatous matrix remains unstained while cartilaginous ground substance is quite strongly impregnated.

I am indebted to Dr. A. D. Morgan, of the Westminster Hospital, for providing me with material from the necropsy on Case 1.

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CONGENITAL TOXOPLASMOSIS

BY

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Since congenital toxoplasmosis was first recognized as a human infection 18 years ago, it has been found in many parts of the world, and, following the first report by Jacoby and Sagorin in 1948, over 50 cases have been recorded in the British literature. Only four of the reports from this country concern the findings in fatal cases (Cathie and Dudgeon, 1949; Valentine, 1952; Valentine, Lane, Beattie, and Beverley, 1953; Morris, Levin, and France, 1955). The clinical picture of the congenital disease, with its characteristic tetrad of hydrocephalus, choroidoretinitis, convulsions, and intracerebral calcification, is now well known, but it is probable that the disease may still pass unrecognized as a cause of obscure oculocerebral defects or perinatal death.

Two cases are reported. The first emphasizes the remarkable pathology which may be found in the acute infection.

Case 1

D. T., a boy, was born by normal delivery at 35 weeks' maturity, weighing 6 lb. Both parents (examined subsequently) are healthy. The only other child, aged 3 years, has a ventricular septal defect but is otherwise well.

The mother had a threatened miscarriage with blood-stained vaginal discharge at the third month of pregnancy, and a slight brown discharge continued for the next six weeks. The pregnancy was otherwise uneventful. The mother had no symptoms of intercurrent infection except for a heavy cold with mild cough during the last seven days before delivery. She recalled no unusual insect bites, and had never been abroad. The father and sister had had no recent illness. The family had never possessed a cat, dog, or any other pet, nor shared a building with any animals.

At birth the child's condition was poor and his temperature unstable, readily becoming subnormal. He was irritable and hypertonic, while the skin was dry and wrinkled as seen in infants born postmaturely. The head circumference was 13 in., though it appeared large owing to the small size of the eyes. The anterior fontanelle was tense and large, admitting three fingers, and a right facial weakness was present. Respirations were grunting and irregular with crepitations at both lung bases. The abdomen was distended and tense and the liver extended two fingerbreadths below the right costal margin.

On the evening after delivery the baby had a convulsion, for which he was given phenobarbitone. His condition remained very poor, with hypertonia and occasional apnoeic attacks. He developed signs of pneumonia in the right lung which improved with penicillin, but continued to show cerebral irritability. A lumbar puncture revealed sterile blood-stained xanthochromic fluid containing 12,500 red cells and 41 white cells (39 lymphocytes, 2 polymorphs); the protein content was 4,000 mg. per 100 ml. (globulin +++) and glucose 123 mg. per 100 ml.

At the age of 19 days it became apparent that the head circumference was increasing and the left parietal bone was bulging. As the fontanelle was still very tense, subdural taps were performed and 20 ml. of cloudy, frothy yellow fluid was aspirated from beneath the left parietal bone. This fluid contained 3,000 red cells, 320 white cells (72% polymorphs, 28% lymphocytes), protein 2,000 mg. per 100 ml. (globulin +++), chlorides 750 mg. per 100 ml., sugar 142 mg. per 100 ml.; no organisms were seen or cultured. Daily subdural taps yielded decreasing volumes of fluid. His condition deteriorated with frequent convulsions, and death occurred at the age of 22 days.

Necropsy Findings.—The body weighed 5 lb. 8 oz. and internal examination showed the liver and spleen to be unusually congested, but no other abnormality was detected in the extraneural viscera. On opening the skull, a pale yellow, thickly gelatinous subdural exudate was found extending over the parietal, temporal, and occipital lobes and filling the cisterna basalis. This substance was adherent to the brain in varying degree, and was up to 7 mm. thick in the parietal regions. The cerebral convolutions were flattened and the posterior parts of both hemispheres felt cystic owing to internal hydrocephalus.

The dilated lateral ventricles contained lemon-yellow fluid and had a shaggy lining of necrotic brain tissue; there was no visible vestige of the choroid plexus. This colliquative necrosis extended to within a few millimetres of the surface of the occipital and temporal lobes, which were cystic with only a narrow shell of relatively intact cortex. The corpus callosum was thinned and the frontal lobes showed necrosis inferomedially in relation to the anterior horns. The basal ganglia were not grossly involved. The third

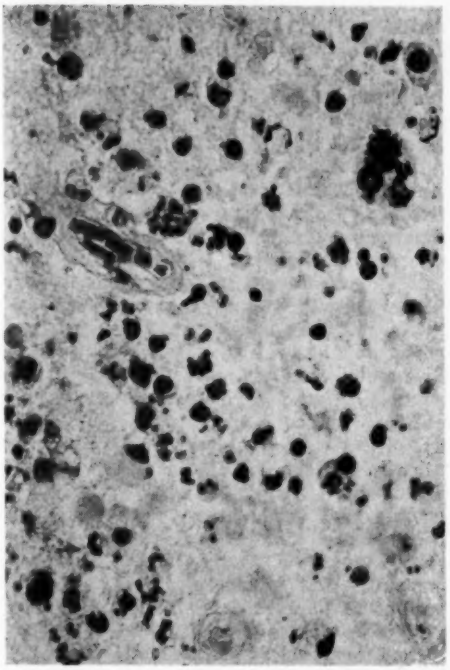

FIG. 1.—Brain tissue showing intracellular and free forms of toxoplasma. Haematoxylin and eosin, × 500.

ventricle was distended downwards to form a sac filled with translucent gelatinous material. There were minute cystic spaces containing similar material at the site of the upper end of the aqueduct of Sylvius, but the remainder of the duct could not be defined.

The fourth ventricle, cerebellum, and cervical cord appeared normal. The whole brain was soft and contained no calcareous material.

Histological Findings.—Sections from the cerebral hemispheres showed severe necrosis with relatively little inflammatory reaction. Immediately adjoining the lateral ventricles there was massive cellular debris with total loss of structure. In marginal areas the necrosis was patchy and at one or two points appeared to have a perivascular distribution, with small round-cell and large mononuclear infiltration, but this distribution was nowhere conspicuous. There was also a variable meningeal reaction.

In one localized area of the hippocampus, large numbers of nucleated bodies with the typical appearance of toxoplasma were found. These occurred extracellularly, both singly and in small groups, and intracellularly as proliferative bodies containing up to 80 individual nucleated structures (Figs. 1 and 2). The debris in other areas of the cerebrum included similar single nucleated structures in varying stages of degeneration, but none would have been identifiable in the absence of the proliferative forms.

The outwardly normal basal ganglia were found to contain extensive irregular granulomatous and necrotic area, with diffuse plasma-cell and small round-cell infiltration. Numerous toxoplasma organisms were present singly and in small groups.

The midbrain and pons were similarly involved, but here no organisms could be found. Necrosis was maximal in the region of the aqueduct of Sylvius, which was obliterated by debris.

The cerebellum was histologically normal except for a slight meningeal reaction in which mononuclear cells were conspicuous. The orbits were not examined histologically.

Serological Findings.—Sera from the family were tested for toxoplasmosis 14 weeks after delivery and both father and sister were negative. The mother's serum was positive to the dye test at a dilution of 1:64 and to the complement fixation test at 1:16. These findings are significant of a recent infection (Cathie, 1955).

In the cerebral type of congenital infection, writers have commented on the congestion of the liver and spleen as the only visceral abnormality, and the failure to find the organism in extraneural tissues. In the rarer forms of congenital infection the presenting neonatal features may include jaundice with hepatomegaly and lymphadenopathy. There seems little doubt that the susceptibility of foetal nervous tissue is at least partly due to the poor diffusion of maternal antibody to this region.

FIG. 2.—Intracellular proliferative body. Haematoxylin and celestin blue, × 1,200.

The intensely necrotic nature of the cerebral lesion and its distinctive and symmetrical distribution gave the impression of an explosive reaction extending outwards from the choroid plexuses and ependyma. To explain this paradoxical distribution of necrosis in areas probably more accessible to maternal antibody, Frenkel (1949) has postulated the formation of a destructive antigen-antibody complex in cells in proximity to the known high antigen content of ventricular fluid.

A characteristic feature of the cerebrospinal fluid is the xanthochromia and very high protein content, with relatively few leucocytes as in Case 1. The absence of intracerebral calcification in this patient is less usual.

Discussion

Mode of Transmission. — Toxoplasmosis is widely distributed in the animal kingdom and has been found in the excreta, saliva, milk, and insect parasites of infected animals. In theory, therefore, numerous avenues are open for the transmission of infection to man.

The increased incidence of positive serological reactions found in those who handle animals, particularly rabbits (Beverley, Beattie, and Roseman, 1954), shows that infection is frequently acquired by animal contact. However, elucidation of this mode of transmission has been difficult. Infection is achieved by cannibalism in starving mice (van Thiel, 1949); thus cats, dogs, and swine would seem to be liable to infection by ingestion of raw flesh, although this proves difficult to demonstrate. Feeding experiments using infected excreta in a variety of animals have also failed, and the natural spread of infection among puppies observed by Olafson and Monlux (1942) stands as an isolated finding among many similar studies. In dogs, as in man, susceptibility appears to diminish rapidly with age, and even in puppies artificial infection requires massive parental inoculation (Jacobs, Melton, and Cook, 1955). There have been a few undoubted instances of human infection in households harbouring infected dogs (Cole, Prior, Docton, Chamberlain, and Saslaw, 1953) and it is interesting that dogs may seem outwardly healthy in the presence of extensive gastro-intestinal ulceration.

Contamination of skin abrasions is possible, since recent conclusive reports of accidental laboratory infection by pin-prick have appeared (Beverley, Skipper, and Marshall, 1955; Ström, 1951), while Sabin, Eichenwald, Feldman, and Jacobs (1952) have reported human infection following a rabbit bite. Sanger, Chamberlain, Chamberlain, Cole, and Farrell (1953) have demonstrated the disease in cattle and the infection of their milk. Biering-Sørensen has been quoted by Siim (1955) as having found the organism in hen's ovaries, with the possibility of infection of raw eggs. Prolonged infectivity has been demonstrated in certain dog ticks (Woke, Jacobs, Jones, and Melton, 1953) but with a low individual incidence, while in other blood-sucking insects the toxoplasma do not survive digestion of the blood clot.

No life cycle has been discovered in insects or animals. *Toxoplasma gondii*, in the only known forms, is an obligate intracellular parasite, rapidly killed by gastric juice and with a low degree of viability outside the body, except in the pseudocystic form found in chronically infected tissues. Thus it would appear that remote contamination of water, milk, or foodstuffs is an unlikely factor in human infection and that airborne infection would require coarse droplet inhalation. However, pulmonary lesions may be found in dogs, cats, and swine, and in man upper respiratory symptoms occur, although detectable lung disease has only been found in severe cases exhibiting various other features.

The majority of acquired cases in man are symptomless and the factors leading to the breakdown of this apparent symbiosis are unknown. Study of the earliest clinical features might be expected to throw light on the usual mode of infection. A form of glandular fever is the most frequently recognized manifestation, and it is to be noted that sore throat is often an initial symptom and glandular enlargement may be predominant in, or confined to, the cervical region (Gard and Magnusson, 1951; Skipper, Beverley, and Beattie, 1954; Cathie, 1954a and b; Siim, 1955). Alexander and Callister (1955) have described a patient with cervical adenitis due to infection at the sixth month of pregnancy, isolating the toxoplasma by gland biopsy. Ström (1951) reported laboratory infection, thought to be acquired by pipette, in which cervical adenitis occurred early in a generalized disease. Oropharyngeal infection is highly probable in such instances, the pattern of local lymphadenopathy followed by dissemination being similar to that which has followed accidental pin-prick.

It is evident, therefore, that the widespread contact of man with toxoplasma may occur in a variety of ways, although upper respiratory episodes may well prove to be a common manifestation of primary infection in spite of the poor viability of the organism.

The Infection in Pregnancy.—Attempts have been made to correlate various untoward events in pregnancy with the onset of infection. Prolonged

and intense lassitude (which may also be a feature of the glandular syndrome) was reported in four cases by Magnusson and Wahlgren (1948). Others have reported a maculo-papular rash (Riley and Arneil, 1950), alimentary disorder, cramp-like abdominal pains, and vaginal haemorrhage (Valentine *et al.*, 1953; Alexander and Callister, 1955). A history of insect bites has been elicited (Wilson and Smith, 1949; Valentine *et al.*, 1953). Holmdahl (1953) found bronchitis (with normal radiological appearances) to coincide with serologically proved infection in mid-pregnancy, and in a second case recurrent pharyngitis was present and there was contact with young birds which later died. Heavy colds in the last weeks of pregnancy have been reported more than once, as in the case recorded here. Nevertheless, all these events could be coincidental, and only in the case of the glandular syndrome has a direct association with toxoplasmosis in mother and child been established.

The importance of infection in pregnancy has been assessed on a large scale by Holmdahl, who showed, as did Gard and Magnusson, that even a proven onset of toxoplasmosis in the mother may not affect the child in any way. He also concluded that, if toxoplasmosis is a cause of abortion, it is a very rare one.

The antibody titre bears no clear relation to the clinical picture, nor is it a reliable indication of the protection afforded to the foetus in pregnancy. It does appear, however, that persistence of antibodies offers some immunity against reinfection or reactivation. The suggestion of Weinman (1952) that chronic latent infection might adversely affect any pregnancy is not supported by the available evidence of animal investigations and clinical experience. No case of congenital infection of successive siblings has been reported, and it appears that transplacental infection only occurs with acute infection in pregnancy.

Case 2

M. B. was a first-born male infant of young healthy parents, and born by normal delivery at 41 weeks' maturity, weighing 6 lb. 10 oz. The mother had had an attack of cramp-like abdominal pain in the right iliac fossa when three months pregnant, with no associated vaginal loss. A few weeks later her parents, with whom she was staying, had an influenza-like illness, but she herself had no respiratory symptoms throughout pregnancy. Her Alsatian dog was healthy during her pregnancy but had diarrhoea when the child was 8½ months old. The mother had no other contact with animals.

The child was spastic and microcephalic from birth. He refused to suck and developed severe ulcerative stomatitis on the third day, associated with a discharge from both eyes. When 4 weeks old he had a series of convulsions which persisted for a week, and thereafter remained spastic with frequent jerks and occasional convulsions.

On admission to hospital at the age of 9 months he was found to be a microcephalic idiot with marked microphthalmos, a small snuffly nose, and large ears. The head circumference was 12½ in. and the anterior fontanelle closed. All the limbs were spastic, with flexion of the arms and hands, extension of the legs, and frequent jerking movements of the whole body. The fundi showed slight pallor of the discs but no choroidoretinitis, and no other abnormalities were found on physical examination. Skull radiographs showed no calcification. The blood Wassermann reaction was negative. The cerebrospinal fluid was blood-stained and contained 128,000 red blood cells, 90 leucocytes (58% polymorphs, 33% lymphocytes), protein 400 mg. (globulin ++), and sugar 63 mg. %.

The baby's condition deteriorated rapidly, and death occurred four days after admission.

Necropsy Findings.—The gross and microscopic findings were those of septicaemia associated with a left-sided pyonephrosis. The spleen was congested and the liver showed severe toxic vacuolation. A left-sided diaphragmatic hernia was present and contained small intestine.

The brain showed atrophy of the convolutions, especially in the frontal and temporal lobes, where the cortex had a coarse, granular appearance, and there was a corresponding excess of very faintly yellow cerebrospinal fluid, which was cloudy and gelatinous in the anterior sagittal fissure and around the temporal poles. Dissection of the brain and orbits, including the ependyma and choroid plexuses, revealed no other abnormality. The ventricles were of normal size. Histological examination revealed atrophy of the cortex with a slight excess of glial cells in the deeper layers. There was no meningeal reaction. No organisms were found in sections of the brain or viscera, nor in wet films of the gelatinous cerebrospinal fluid and smears from adjoining areas of the temporal lobes.

Serological Findings.—Serum from the Alsatian dog and the baby's serum at 9 months of age were negative for toxoplasmosis in both dye and complement fixation tests. The mother's serum was positive to a titre of 1:256 in the dye test and to 1:64 in the complement fixation test. These tests were repeated in the mother six weeks later and showed a considerable fall in titre, the dye test being 1:80 and the complement fixation test 1:4.

There is no positive evidence that this child's condition was due to toxoplasmosis, although if this were the case it would represent an early burnt-out infection in which, with negative serology, one would not expect to find surviving organisms. The mother had undoubtedly reacted to a recent infection, but this could have been acquired after the birth of the baby.

Summary

The clinical and pathological features of a fatal case of congenital toxoplasmosis are described, and an example is also given of a microcephalic infant whose mother showed evidence of a recent infection with toxoplasmosis. The mode and significance of maternal infection are discussed.

Although it appears that the possible paths of infection are multiple, there is some evidence in favour of the oropharyngeal route. The only manifestation likely to give rise to the diagnosis in pregnancy is the glandular syndrome.

Transplacental transmission has only been shown to occur as a result of infection acquired during pregnancy, and even in this event the degree of hazard to the foetus cannot at present be assessed.

The importance of toxoplasmosis in relation to mental deficiency, microcephaly, and ocular defects may be greater than is revealed by current diagnostic criteria. The absence of serum antibodies after the first few months of life does not exclude a past infection *in utero*.

We wish to thank Dr. I. A. B. Cathie for carrying out the serological investigations.

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LYMPHADENOPATHY IN TOXOPLASMOSIS

BY

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The clinical signs of congenital toxoplasmosis are now well known, but those of post-natally acquired infections have received less attention. Sabin (1941) recorded two cases which presented as meningoencephalitis, and Pinkerton and Henderson (1941) described two cases which resembled typhus.

In 1951 Siim reported seven cases which had generalized lymph node enlargement not unlike glandular fever. Since then many other cases of lymphadenopathy have been described (see Siim, 1956).

The latter type of illness appears to be much commoner than the others described. In most cases there have been abnormal cells of the lymphocyte-monocyte series in the peripheral blood and in none of them has the Paul-Bunnell test been positive. In only a few cases have lymph nodes been submitted to histological examination, and the purpose of this report is to describe a case in which the histological appearance resembled that described by Robb-Smith (1947) as lympho-histiocytic medullary reticulosis.

Case Report

Miss I. P., aged 26 years, attended the Medical Out-Patient Department on August 13, 1956. She had been in good health until three days previously when she developed throbbing occipital headaches present on waking and continuous throughout the day. She also complained of a constant, dull low back pain radiating into the back of both thighs. Her symptoms were aggravated by change of posture and caused loss of sleep. Until two weeks before her illness she had been living in York where she was employed as a Government driver. Her work involved visits to outlying farms and she was in close and frequent contact with animals.

Examination revealed an intelligent and well-nourished woman. She was flushed but afebrile. The conjunctivae were of good colour and there was no skin rash. The fauces were clear and no palpable lymph nodes were detected. The heart and lungs were within normal limits at clinical examination. The liver and spleen were not palpable. In the central nervous system the optic fundi showed no abnormality and the cranial nerves were likewise normal. There was no neck rigidity, but flexion of the cervical spine produced some minor discomfort. Straight leg raising was full, but produced some pain in the lumbar spine. The tendon reflexes were present and equal. The plantar responses were flexor. Muscle power was normal and there were no other abnormalities.

The possibility of a non-paralytic poliomyelitis or a benign lymphocytic meningitis was considered and the patient was admitted to hospital for further investigations. The lumbar puncture produced a clear fluid under a pressure of 200 mm. of water. Laboratory investigations gave the following results:

Cells, 5 lymphocytes per c.mm., protein, 70 mg./100 ml., globulin, negative, chlorides, 620 mg./100 ml., glucose, 62 mg./100 ml.

The patient remained afebrile and her symptoms subsided within a few days.

She was eventually discharged to the Out-patient Department after 14 days in hospital. She was seen again four weeks later on September 24, 1956, when she stated that some days after returning home she again began to feel ill. On about September 10 there had been a recurrence of low backache and headache, and at the same time she noticed tender swellings in the right groin, followed a few days later by further swellings in the right axilla and in the neck.

Examination showed a widespread lymphadenopathy with discrete, tender glands palpable in the left posterior cervical chain, and a soft, tender gland in the left supraclavicular fossa some ¾ in. diameter, with similar glands in the right axilla and right groin.

The only other physical sign was a marked tenderness over the lumbar spine and the left sacro-iliac joint.

Within a few days the glands began to subside, but the patient continued to complain of severe pain over the lumbar spine and persistent headache.

Blood investigations were within normal limits, and lymphatic gland biopsy was carried out on October 17, glands being removed from the right axilla and the right inguinal region. The biopsy specimen revealed a histological picture compatible with so-called lympho-histiocytic medullary reticulosis (Robb-Smith, 1947), and it was decided to continue symptomatic treatment and to follow her progress as an out-patient. The lymphadenopathy continued to subside, and she was allowed home on November 3 with instructions to report for review in a further two weeks.

During this interval it occurred to us that she might be a case of toxoplasmosis and when next seen on November 19 blood was taken for serum antibody tests. Strongly positive dye tests for toxoplasmosis were obtained together with a positive complement-fixation test. Previous samples of sera were examined in retrospect for evidence of toxoplasmosis and a progressively rising titre diagnostic of acquired toxoplasmosis was found (Table I).

TABLE I
TOXOPLASMOSIS DYE AND COMPLEMENT-FIXATION TESTS

Date	Dye Test	Complement-fixation Test
17/8/56	1/77	Negative
23/8/56	1/102	
19/11/56	1/4,600	1/20
12/12/56	1/12,000	1/20

The patient continued to make steady progress and when last seen in January, 1957, was well and symptomless, and the lymphadenopathy had completely subsided.

Pathology

Two lymph glands were received for examination, one from the right inguinal region and one from the axilla. That from the inguinal region measured 20 mm. in diameter, and that from the axilla 9 mm. diameter.

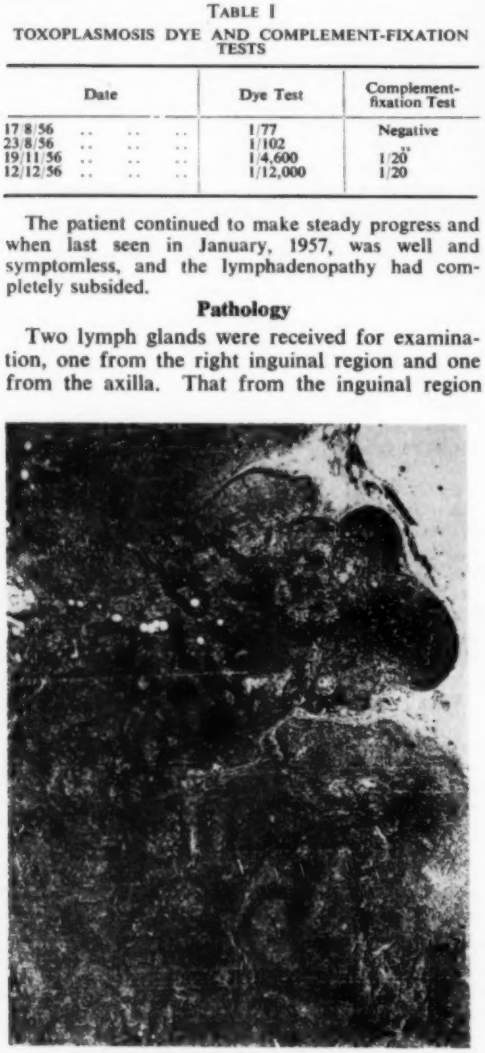

FIG. 1.—Exaggerated follicular pattern in an axillary gland. Haematoxylin and eosin, ×90.

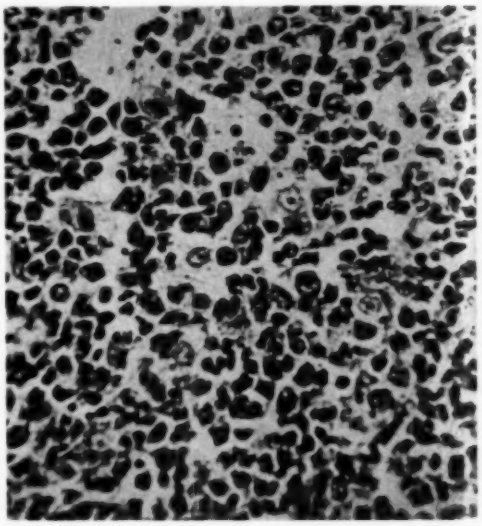

FIG. 2.—Scattered epithelioid cells in an axillary gland. Haematoxylin and eosin, ×380.

The histological appearances of both were essentially similar. The follicular pattern was still present in the axillary gland (Fig. 1), but this was less evident in that from the inguinal region. Very striking were medullary collections of mono- and multinuclear epithelioid cells with an eosinophilic cytoplasm. There was no caseation and no false follicle formation, but in some areas these collections of epithelioid cells showed a sarcoid-like pattern (Fig. 2). Serial sections of the glands did not show any toxoplasma pseudocysts.

Discussion

In general the picture was similar to that described by Robb-Smith (1947) as lympho-histiocytic medullary reticulosis. It seems that this condition is a reactive one, although Robb-Smith (1947) reported a survival rate of 95% after one year and 5.5% after 10 years.

The histological appearances in the lymph glands in toxoplasmosis have been described by other observers. Some of these changes are also similar to those described as lympho-histiocytic medullary reticulosis.

Stanton and Pinkerton (1953) reported a diffuse reticulosis, on first impression suggestive of malignant lymphoid tumour. They noted that the follicular architecture was preserved, and stressed

the presence of focal collections of eosinophilic histiocytes. In their case, by cutting serial sections of the glands, toxoplasma pseudocysts were found.

Skipper, Beverley, and Beattie (1954) described the appearance as being very similar to that seen in infectious mononucleosis. Small rounded groups of large cells were noted scattered through the gland pulp. Some of these were multinuclear.

Wahlgren (1951) reported the condition as subacute lymphadenitis with swelling of reticulum cells and agglomeration of eosinophilic leucocytes and plasma cells in the tissue. Other observers, however, have not stressed this medullary reaction in the lymph glands.

Siim (1952, 1953, 1954–5) and Gard and Magnusson (1951) discuss a mainly follicular lymphoid hyperplasia.

It appears that the changes in the lymph nodes in toxoplasmosis, whilst commonly reactive and non-specific in nature, can, when the disease is well established, be more characteristic. It is suggested, therefore, that toxoplasmosis be considered in any transient lymphadenopathy where the diagnosis remains obscure and especially where the histological appearances of the gland are of a non-specific or reactive character. It would not appear possible, however, to make the diagnosis by histological examination alone, except in those cases where the parasites are actually demonstrable. Serological dye and complement-fixation tests are necessary.

Summary

A case of lymphadenopathy is described in which extreme reactive changes, resembling lympho-histiocytic medullary reticulosis, were found to be due to toxoplasmosis.

We wish to thank Dr. C. S. Darke for permission to publish this case, and we gratefully acknowledge the advice of Professor C. P. Beattie and the technical assistance of Mr. C. Lambourne.

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PRIMARY MELANOMA OF THE LEPTOMENINGES

BY

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Primary melanomata of meninges are rare tumours, and well-documented cases rarer still. In 1935 Akelaitis, in a comprehensive review of the literature, collected 29 cases and added one of his own. In 1949 Touraine reviewed all the reported cases of primary pigmented tumours of the meninges; the total was then 66, an addition of only 29 cases in 14 years. Both Akelaitis and Touraine, however, express doubt as to the validity of a number of the reported cases, and Touraine accepts only 27 as being true malignant primary melanomata of the meninges. In that number are included six cases, namely, those of Pol (1905), Weimann (1923), Ehnmark and Jacobowsky (1926), Baumecker (1929), Mackay and Hurteau (1942), Foot and Zeek (1931), case 2, which are unacceptable, as pointed out later, because of the presence of extracranial tumours. Since then six more reports have appeared (Strong, 1947; Pautler and Gallavan, 1951; King, Chambers, and Garey, 1952, two cases; and Russi, Robinson, and Nagler, 1953, two cases). Both the cases of Strong and of Pautler and Gallavan revealed extracranial tumours at necropsy, while Case 2 of Russi *et al.*, as the authors themselves imply, does not fulfil the criteria for the diagnosis of a primary melanoma of the meninges. We thus have a total of only 24 cases in which a diagnosis of primary malignant melanoma of the meninges is considered acceptable.

The purpose of this paper is to record another case which, it is believed, is the first one to have been diagnosed in life in Great Britain.

Clinical History

A. S., a man aged 25, in August, 1955, woke up with pain in the occipital region, the back of the neck, and radiating down the spine. He vomited and was unsteady on his feet. At the hospital where he was admitted the only reported abnormality was in the cerebrospinal fluid, which contained 23 lymphocytes/c.mm. and 300 mg. protein per 100 ml. He was transferred to the Regional Neurosurgical Unit.

On admission on November 2, 1955, he was confused, euphoric, occasionally having hallucinations and unable to stand because of paroxysms of disequilibrium. His wife reported that during the past three months his memory had deteriorated and he had become irritable. He had slight papilloedema on the left and fine tremor of the outstretched hands, but otherwise no neurological signs. A lumbar puncture, performed on November 7, showed that the cerebrospinal fluid contained 28 lymphocytes/c.mm. and 350 mg. protein per 100 ml. The sugar content was 50 mg. per 100 ml. and chloride 690 mg. per 100 ml. No malignant cells could be seen. Radiographs of the skull and chest were normal. Ventriculography revealed markedly enlarged symmetrical ventricles with the aqueduct and the fourth ventricle patent. A provisional diagnosis of arachnoiditis was made with a block at the tentorial level. In view of his continuing deterioration it was decided to explore the posterior fossa.

At operation on December 13, on opening the dura the leptomeninges covering the cisterna magna and brain-stem were seen to be uniformly and densely covered with dark brown and black deposits; a biopsy was taken. The biopsy material (Fig. 1) showed a diffuse infiltration of the meninges by irregular groups and sheets of heavily pigmented cells. The reactions of the pigment were those of melanin. Nuclear details were mostly completely obscured by the pigment, but there were some bizarre forms and binucleated cells. After operation and decompression, his symptoms temporarily abated and he was discharged home on January 4, 1956.

The diagnosis was melanosis of the leptomeninges.

Three days after discharge the patient was readmitted because of retention of urine. His general condition gradually deteriorated and he died on January 16.

Necropsy.—The skin was deeply pigmented all over, somewhat reminiscent of a suntan, but his ancestry was English and he had not been sunbathing. No abnormality was found except in the cerebral nervous system, and no neoplasm was found in any other viscus. There were no moles or previous operation scars of any sort. All the nails were carefully examined, and no subungual melanoma was found. The eyes were taken out for microscopical examination.

FIG. 1a.—Biopsy of leptomeninges, showing meninges infiltrated by irregularly pigmented cells. Notice tendency to perivascular distribution of pigmented cells.

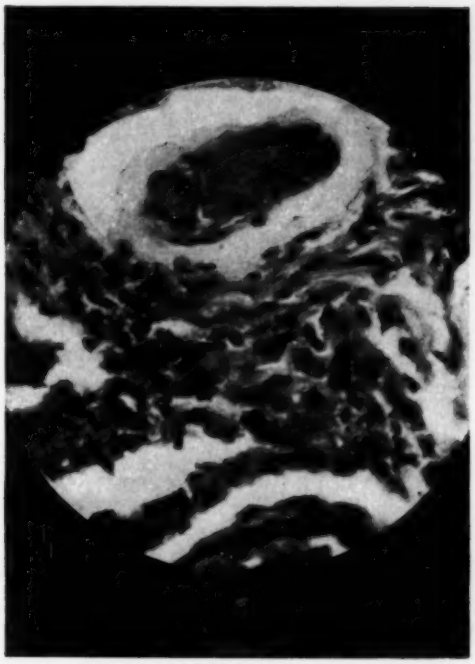

FIG. 1b.—High-power view of Fig. 1 (×400) to show large bizarre cells and irregular nuclear staining.

Central Nervous System.—The leptomeninges covering the base of the brain and its under surface (Fig. 2) were jet black, the pigmentation being thickest in the midline stretching from the medulla to the optic nerve, obscuring the optic chiasma. Laterally the pigmentation spread out more or less symmetrically on the inferior surfaces of the cerebellum and of the temporal lobe, and tended to follow the lines of the main fissures, especially the Sylvian fissure. Anteriorly it spread along the olfactory tracts: as the pigmentation thinned out from the main mass it became more discrete and resolved into flat miliary nodules of about 1 mm. in diameter. All the issuing nerves, especially the optic chiasma and nerves, were ensheathed, but apparently not invaded. As it was decided to preserve the specimen serial coronal sections were not made, but the upper surface of the parietal and occipital lobe on one side was shaved to reveal the lateral ventricle (Fig. 3). The ependyma was seen to be covered uniformly by the same miliary nodules.

The circumferential pigmentation of the brain-stem continued uninterrupted along the whole length of the spinal cord (Fig. 4) and the separate roots of the cauda equina received their separate coats. Sections of the cord at various levels again showed the pigmentation to follow the fissure, but no infiltration of the cord was apparent. The dura was normal.

Histology.—The eyes were normal. The whole of the leptomeninges was widely infiltrated by pigmented cells. The pigment gave the characteristic reactions of melanin. The arrangement of the cells was variable, but mostly they lay in loosely knit sheets with very scanty supporting stroma. In the denser areas there was a tendency to form syncytium, but otherwise the cells were distinct; perivascular cuffing was a notable feature, and indeed here and there the cells were carried into the nervous tissue substance for a short distance through the perivascular spaces, although there was no direct invasion of the nervous tissue. The cells were mostly polygonal or oval. The pigmentation was very irregular and uneven, and, where the degree of pigmentation allowed detailed microscopy, the nuclei were seen to be vesicular with a dense nuclear membrane and a well-defined nucleolus; giant and binucleated forms were present; the degree of pigmentation sometimes did not allow differentiation from pigment-laden histiocytes, but in the lighter areas the cells were obviously neoplastic (Fig. 5).

Nervous Parenchyma.—As noted above, direct invasion was not observed, but all the spinal roots of

FIG. 2.—Base of brain showing distribution of pigment.

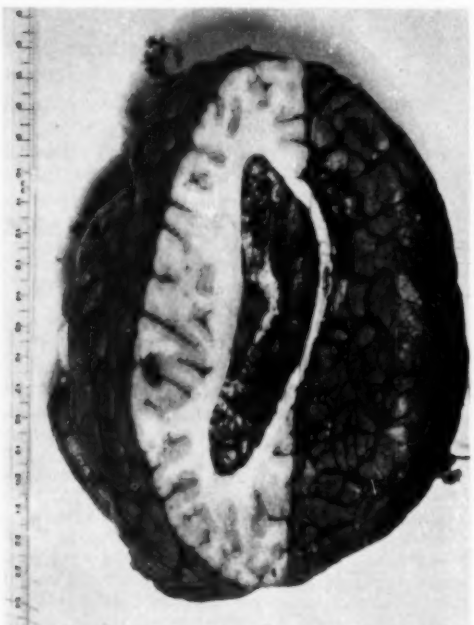

FIG. 3.—Cavity of lateral ventricle viewed from above.

FIG. 4.—Spinal cord and cauda equina.

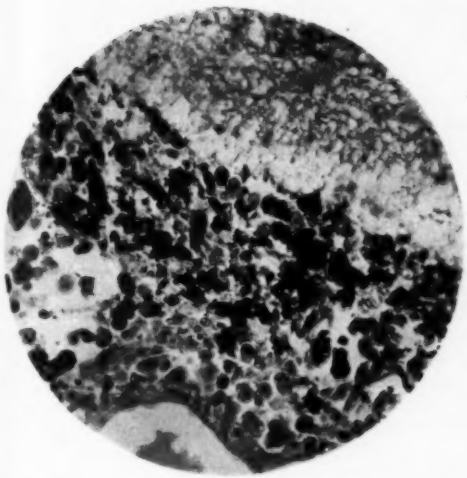

FIG. 5.—Leptomeninges covering pons (× 320) showing irregular pigmentation; lighter areas show irregularity of shape and size of cells.

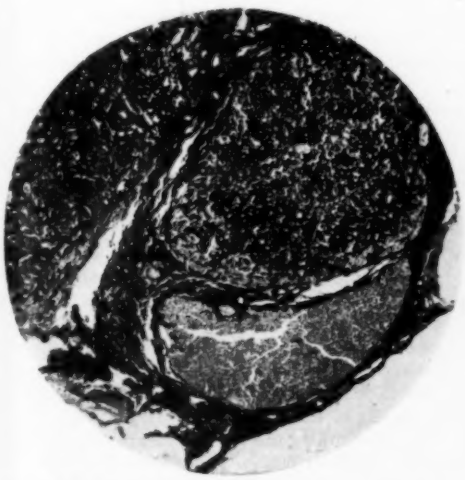

FIG. 6.—Spinal nerve (× 80). The nerve bundles are closely invested in a sheath of pigmented cells following the line of the fibrous tissue septa between the fibres.

peripheral nerves were closely invested in a mantle of pigmented cells, and these also followed the fibrous tissue septa lying between the constituent fibres (Fig. 6).

Optic Chiasma.—This lay in the densest zone of pigmentation. Again no nervous invasion could be seen, but the nerve fibres were ensheathed by the neoplastic cells, some of which had penetrated the glial coverings.

Wall of Ventricle.—A thin layer of pigmented cells lay among the ependymal cells; there was no extension into the white matter and no destruction of either ependyma or nerve cells.

The pituitary was normal.

Conclusions and Comments

There is no doubt that this case is one of primary melanosis of the leptomeninges. The question of malignancy is more difficult to evaluate. No definite evidence of invasion could be found, but the irregular proliferation of the cells, the irregularity of their pigmentation, the presence of bizarre and giant forms leave no doubt that malignant change had taken place *in situ*. This is further supported by a comparison with the case of Forbes and Maloney (1950). The latter resembles the present case in all its aspects and histological details, with the exception that Forbes, in addition, demonstrated direct invasion of the spinal cord in one area. It follows that malignant change can occur elsewhere along a neuraxis which is already the seat of a widespread melanosis and can occur at several places simultaneously. Multicentricity of origin must not be misinterpreted as dissemination from a primary focus.

Therefore the points which should always be considered in cases of primary meningeal melanomata are (*a*) the diffuse melanosis, (*b*) the absence of tumours elsewhere, and (*c*) the associated skin pigmentation.

(*a*) In the majority of reported cases of melanomata there has been a diffuse infiltration of the meninges. Indeed, Winkelman, Gotten, and Silverstein (1936) consider that one of the diagnostic criteria of primary melanoma of the meninges is their diffuse involvement. King *et al.* (1952), however, point out that, although this may be the final situation as seen at necropsy, the early stages (as in their case 1) may be a localized tumour of the meninges, which later on disseminates along the neuraxis. Be that as it may, a strictly localized tumour, without accompanying melanosis, especially one causing destruction of bone, should arouse suspicions of a primary elsewhere or of a pigmented meningioma. Kissel, Rousseaux, Beau, Midon, and Arnould (1950) report such a case of localized "primary" melanoma of the meninges which recurred after excision, but no necropsy report was included and a large melanoma of the foot was present on which there was no histological report. Such cases warrant fuller documentation and will again be discussed.

A more difficult problem is that of the pigmented meningioma. Ray and Foot (1940) first drew attention to the resemblance between localized "melanomas" and meningiomas, and Bakody, Hazard, and Gardner (1950) report a case where a mass, localized to L2–L4, was excised and diagnosed as a melanoma. Six years after the operation, as the patient was still well, the slide of the previous operation specimen was reviewed and the tumour was found to be a meningioma. In an analysis of 58 cases of melanotic tumours of the meninges, Touraine (1949) found a diffuse melanosis in 17 out of the 27 cases in which there was indubitable evidence of malignancy, either invasion of the nervous parenchyma or dissemination. The remaining 31 he regarded as simple melanosis, although recognizing two degrees of the condition, a diffuse form (*melanisme*) of which there were eight cases, and a more discrete form (*melanose*) of which there were 22 cases. It would not, therefore, be unreasonable to assume that melanosis, diffuse in the majority of cases, always precedes the development of malignancy, and must be regarded as an essential prerequisite of primary melanomata of the meninges. This would seem more plausible than to regard the melanosis as secondary dissemination as King, Chambers, and Garey (1952) suggest.

(*b*) Melanomata of the meninges are not as malignant as their counterpart in the skin and eyes. Visceral metastases are not found (Willis, 1948). Strong (1947), arguing against this view, quotes a case of Foot and Zeek (1931) and one of his own, in which there were widespread melanotic tumours in the skin, lymph nodes, lung, right adrenal, stomach, and cerebellum. In the presence of so many possible sites for the primary, it seems unwise—to say the least—to select the cerebellar meninges as the source of so many metastases. The case of Foot and Zeek is rejected by Willis on the same ground. Forbes goes further and suggests that all cases in which there is a history of previous operation on a skin or eye tumour should be rejected. Bakody *et al.* (1950) go yet further:

> "A primary leptomeningeal origin cannot be accepted as such unless a careful search fails to disclose a primary malignant lesion in the eye, skin, or visceral organs, and even then the presence of intracerebral melanotic nodules could challenge an interpretation of primary origin."

These criteria seem laudable and exclude the eight cases mentioned in the introduction.

There is no doubt then that in the majority of reported cases the neoplastic process has remained localized to the meninges and the central nervous system. This behaviour, remarkably different from the highly malignant cutaneous tumours, may have some importance histogenetically. Many theories have been put forward to explain the origin of melanomata. It is beyond the scope of this report to embark upon a detailed discussion of this problem.

The majority of pathologists nowadays accept the view that cutaneous melanomata are epithelial in origin, as shown by Dawson (1925). The opponents of that theory, in particular those who still support Masson's views that melanomata are derived from sensory nerve endings, believe that such nerve endings exist in the leptomeninges (Stoler, quoted by Akelaitis, 1935), and would be the origin of meningeal melanomata (Foot and Zeek, 1931). Such a unified conception ignores the above-mentioned difference in behaviour, which suggests a difference in cell potentiality and hence a difference in the cell of origin. Branched, pigmented cells are normally present in the meninges and described in standard textbooks of histology (Maximow and Bloom, 1948). It seems reasonable to assume that they are the cells of origin of melanosis and melanomata of the meninges. Their own origin, whether neuroepithelial or mesoblastic, is more difficult to establish. But this controversy should have no bearing on the theories of origin of cutaneous melanomata.

(*c*) The most interesting feature of the condition is the associated abnormalities of pigmentation in the skin. In an analysis of 42 cases of diffuse melanosis, in which information regarding the skin was published, Touraine found that in 56% there was abnormal pigmentation of the skin and in 26% the abnormality was severe enough to cause disfiguration. (By contrast, in seven cases in which localized primary melanoma of the meninges was diagnosed without associated diffuse melanosis of the meninges, two only had abnormal pigmentation of the skin. These figures are additional evidence that localized "melanoma" without the accompanying melanosis of the central nervous system is a separate entity and probably not a primary disease of the meninges.) It is likely that primary melanosis of the meninges is part of a systematized abnormality, probably congenital, of pigment formation. However, the roles of the endocrines cannot be ignored in view of the well-known disorder in Addison's disease.

The pituitary, too, plays a part in the control of pigment production and extracts of the posterior lobe cause an expansion of the skin melanophore in the frog. In man, the chloasma of pregnancy

may well be attributable to pituitary hyperfunction, while victims of pituitary cachexia may develop a generalized pigmentation. In the present case, however, the endocrines were normal.

Summary

A case of primary melanosis of the leptomeninges is described, and the literature is reviewed.

The criteria for diagnosis are discussed.

It is suggested that melanosis is a form of a systematized abnormality of pigmentation.

I wish to thank Dr. W. Gibb, consultant neurosurgeon, for permission to publish this case, and Dr. I. Williams, pathologist, Brook Hospital, for permission to use material, and Dr. E. N. Allott, Director, Lewisham Group Laboratory, for assistance in the preparation of this paper.

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TRANSFUSIONAL HAEMOSIDEROSIS SIMULATING HAEMOCHROMATOSIS

BY

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Haemochromatosis is a disease occurring predominantly in men, and characterized by excessive deposition of iron in the tissues, coexisting with skin pigmentation and hepatic, pancreatic, and testicular fibrosis. The condition is described by Sheldon (1935) in whose monograph 311 cases are reviewed.

Parenteral iron given in excess of requirements will be deposited in the tissues, since, with the exception of menstruation in women, the body has no normal mechanism for the elimination of significant amounts of iron. Except in haemorrhagic states, similar iron deposition will follow multiple blood transfusions. The distribution of this iron and its effect on the tissues is a study of considerable theoretical interest and of some practical importance. On the existence of a type of haemochromatosis known as acquired, secondary, exogenous, or transfusional, opinion is divided. Schwartz and Blumenthal (1948), Aufderheide, Horns, and Goldish (1953), Goldish and Aufderheide (1953), Morningstar (1955), and Pengelly and Jones (1956) have reported cases described as haemochromatosis caused by multiple blood transfusions. That multiple blood transfusions alone can cause haemochromatosis is denied by Wyatt, Mighton, and Moragues (1950), Cottier (1952), Kleckner, Baggenstoss, and Weir (1954), Kleckner, Kark, Baker, Chapman, Kaplan, and Moore (1955), and by Dubin (1955).

The following case is reported of a woman who, having received 150 "pints" of blood for an aplastic anaemia, developed haemosiderosis seen as pigmentation of the skin and detectable in the sternal marrow and mucosal glands of the stomach. Post-mortem examination showed an extensive haemosiderosis with hepatic and pancreatic fibrosis.

Case Report

A woman of 67 presented with a two years' history of increasing lassitude and pallor. This proved on investigation to be due to an aplastic anaemia. No toxic cause was discovered and the previous medical and family history was not relevant. On examination she was markedly pale. The spleen and liver were just palpable on inspiration. A diffuse systolic murmur was audible throughout the precordium. The haemoglobin on admission was 31%, estimated by the oxyhaemoglobin method (100% = 14.8 g./100 ml.), and the white cell count was 2,800/c.mm., of which 48% were neutrophils. The sternal marrow showed a normal cellularity with relative hypoplasia of the red cell series. Free iron was present in the marrow section but not in excess.

Treatment in the first instance was with iron by mouth, folic acid 15 mg. t.d.s., vitamin B_{12} 100 μg. daily, and tabs. thyroid sicca $\frac{1}{2}$ grain b.d. There was no evidence that any of these agents either singly or in combination had any influence on the blood picture, and throughout her long illness the only successful treatment was that of transfusion of blood given as packed cells, the blood group being O rhesus negative. Transfusions were given at approximately six-weekly intervals and averaged about five bottles of whole blood, given as packed cells each time, for the next three years. (The National Transfusion Service bottle of whole blood contains approximately 540 ml., of which 120 ml. is anticoagulant.) During this time the haemoglobin varied from 25 to 30% before transfusion to 75 to 85% after; the total white cell count was remarkably constant at 2,000–2,500/c.mm. with about 50% neutrophils, and its relatively slight variations bore no relationship to the variations in haemoglobin. Platelets remained at about 100,000/c.mm., and reticulocytes were consistently absent or less than 1%. Ferrous sulphate was given by mouth for the first two months only and did not exceed a total of 50 g. During the last year of her life the transfusions were a little larger and more frequent and the haemoglobin was some 10 to 15% higher.

During the first 12 months of the illness 36 bottles of blood were given. At the end of this time the sternal marrow showed the same picture as on the first admission. Three years from the first admission, and after 110 bottles of blood, pigmentation of the skin became definite, generally of a slaty-brown colour; this was accentuated in the flexures and was particularly marked around the neck. Biopsy confirmed free iron-containing pigment situated in the

dermis. At this time the prothrombin efficiency was 40% of normal, and was the only abnormal finding suggestive of liver involvement; standard liver function tests were normal. Two months later, after 122 bottles, the sternal marrow showed free iron in great excess of normal, and a glucose tolerance test was performed. After 50 g. of glucose by mouth the blood sugar rose from a fasting level of 67 mg./100 ml. to 126 mg. at half an hour, 208 mg. at one hour, and fell slowly to 150 mg. at two hours. To exclude the possibility that this result was due to a low dietary intake of carbohydrate, the patient took a high carbohydrate diet (200 g./day) for a month and the test was repeated with almost identical results. There was no glycosuria during either test. At this time gastric biopsy showed a deep brown pigmentation in the gastric glands; this pigment stained heavily for free iron. From this time onwards she was treated with cortisone, 25 mg./day by mouth for four weeks, then 75 mg. per day. After eight weeks a fall of only 4% haemoglobin in the succeeding four weeks was found. The haemoglobin dropped 35% in the following two weeks, and thereafter the rate of fall of haemoglobin was the same as before this temporary remission. Two months later, 46 months from her first admission and after 146 pints of blood, she suddenly deteriorated; haemoglobin was 90%, but the white cell count rose to 20,000/c.mm. with a polymorphonuclear leucocytosis, and in spite of penicillin therapy she died within three days. The only indication of a source of infection had been a transient urinary infection six months previously. Possibly the cortisone, although in only moderate dosage, had masked the symptoms. The enlargement of liver and spleen found on first admission had not increased. There had at no time been any clinical evidence of chronic heart failure or venous congestion.

Necropsy Findings

Necropsy (P.M. 166/56) was performed on August 10, 1956, 24 hours after death.

The body was that of an elderly asthenic woman showing widespread brownish pigmentation, particularly in the flexures, and numerous bruises.

Cardiovascular System.—The pericardium was roughened and covered by a thick, shaggy exudate. The heart weighed 398 g. The valves and chambers were normal apart from a patent interauricular septum, the aperture measuring 1 cm. in diameter. The myocardium was brown.

Respiratory System.—The lungs were moderately congested. The right upper lobe contained a subpleural abscess 1 cm. in diameter.

Digestive System.—The stomach contained altered blood. The intestines were normal.

Liver.—The liver weighed 1,930 g. The surface was smooth and dark brown as was the cut surface. No obvious nodularity was present. There was no evidence of chronic venous congestion. The gall-bladder and bile ducts were normal.

Pancreas.—The pancreas was dark brown but otherwise appeared normal.

Genito-urinary System.—The left kidney weighed 171 g., the right kidney 199 g. Both showed several cortical abscesses, the largest measuring 1 cm. in diameter. The bladder contained pus and a thick exudate was adherent to the mucosa. The uterus, Fallopian tubes, and ovaries were normal.

Spleen.—The spleen weighed 256 g. It was firm in consistency and dark brown. No enlarged lymph nodes were found in the neck, axillae, or mediastinal, inguinal, or abdominal areas.

Endocrine System.—The thyroid, suprarenals, and pituitary glands appeared normal.

Central Nervous System.—The brain showed slight oedema but was otherwise normal.

Histological Examination

Sections were stained with haematoxylin and eosin and also were treated by Perles' method for the demonstration of iron. Haemosiderin was identified as a brown pigment which gave the free iron reaction. No brown pigment was found that failed to give the free iron reaction and it was deduced that haemofuscin and lipofuscin pigments were absent.

Heart.—The cardiac muscle contained a moderate amount of haemosiderin, mainly within the muscle cells.

Lungs.—The right lung showed an abscess in the upper lobe. The left lung was congested. Traces of haemosiderin were present in the alveolar septa.

Liver.—The liver showed marked fibrosis in the portal tracts, the fibrous septa of adjacent portal tracts sometimes coalescing (Fig. 1). True pseudo lobulation, as found in hepatic cirrhosis, was not seen. A large amount of haemosiderin was present, mainly among the fibrous septa and within Kupffer cells (Fig. 2). Small amounts of haemosiderin were present in the hepatic parenchymal cells.

Pancreas.—The pancreas showed moderate fibrosis (Fig. 3). A large amount of haemosiderin was present in the increased fibrous stroma. Only small amounts of haemosiderin were present in the pancreatic parenchymal cells (Fig. 4). The islets of Langerhans appeared less cellular than normal and scanty haemosiderin was present within the islet cells.

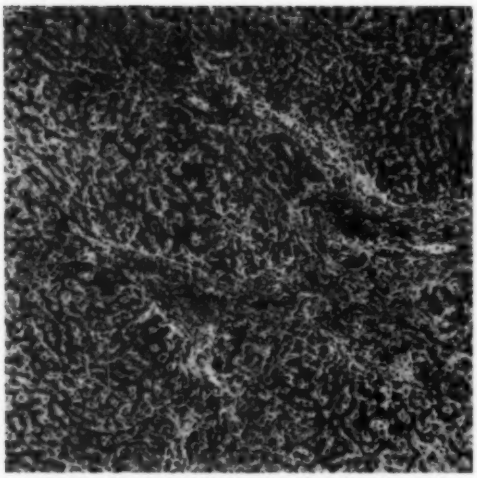

FIG. 1.—Liver stained by haematoxylin and eosin, × 50.

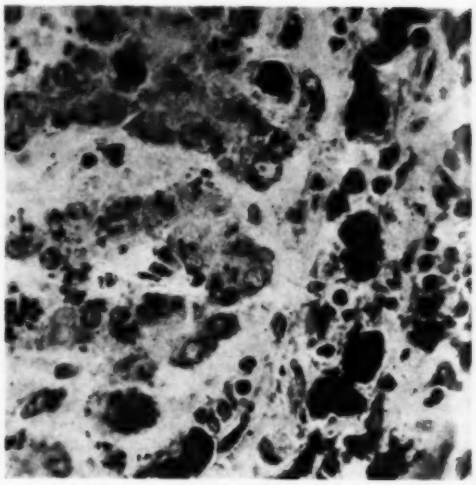

FIG. 2.—Liver stained by Perls' method, × 450.

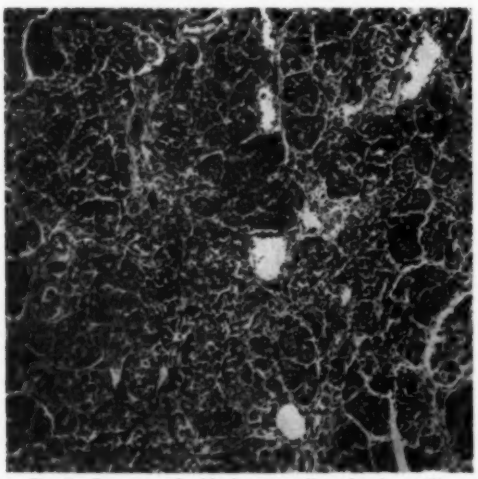

FIG. 3.—Pancreas stained by haematoxylin and eosin, × 50.

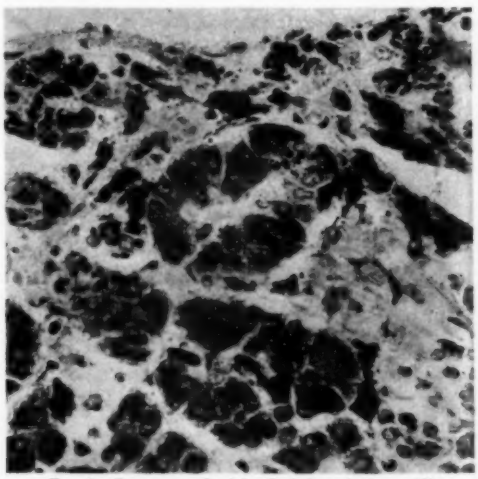

FIG. 4.—Pancreas stained by Perles' method, × 450.

Kidneys. — The kidneys showed cortical abscesses. Small amounts of haemosiderin were present in the connective tissue stroma and within the renal tubular epithelium.

Spleen.—The capsule showed moderate thickening, but the fibrous trabeculae were not prominent. The splenic pulp was very congested. A large amount of haemosiderin was present, mainly within phagocytic cells but also lying free in the reticular framework of the spleen.

Suprarenal Glands. — Moderate amounts of haemosiderin were present, mainly in the parenchymal cells of the adrenal cortex.

Brain.—The brain showed very scanty haemosiderin pigment.

The examination showed an extensive haemosiderosis with moderate hepatic and pancreatic fibrosis. The distribution of iron was widespread, but the greatest amounts were usually in con-

nective tissues and within phagocytes. No lipofuscin or haemofuscin type pigments were found. The ultimate cause of death was pyaemia.

Iron Analyses

The iron content of several of the tissues was estimated by the method of Kennedy quoted by Hawk, Oseir, and Summerson (1947). Estimations were performed on random samples of wet tissue and in duplicate except in the cases of the kidney and brain. The results are shown in Table I, in which figures for normal subjects (Muirhead, Crass, Jones, and Hill, 1949) are included.

TABLE I
IRON CONTENT OF TISSUES

Tissue	Weight of Organ (g.)	Total Iron Content of Organ (g.)	Iron Content (mg./100 g. Wet Tissue)	Iron Content of Normal Subjects (Muirhead) (mg./100 g. Wet Tissue)
Liver ..	1,930	11·6	600	16·6
Pancreas ..	200 (estimated)	1·6	800	7·37
Heart ..	398	0·5	120	—
Spleen ..	256	1·0	400	—
Lungs (both)	1,080	0·5	50	12·87
Muscle ..	—	—	40	—
Kidneys ..	370	0·3	80	10·16
Brain ..	1,190	Less than 0·1	Less than 10	—

Conclusions

The present case shows haemosiderosis with some of the features of haemochromatosis. The latter diagnosis appears unacceptable because of the following features which distinguish this case from haemochromatosis.

Distribution of Haemosiderin.—In this case a large amount of haemosiderin was present in the bone marrow, spleen, and liver, where it was mainly in the portal tracts among fibrous tissue and within Kupffer cells. In haemochromatosis, haemosiderin is not prominent in the bone marrow or spleen and in the liver appears mainly in parenchymal cells. Similarly iron deposition in the pancreas in this case was mainly within phagocytes and free in connective tissue septa.

Mild Degree of Hepatic and Pancreatic Fibrosis.—In haemochromatosis severe portal cirrhosis and pancreatic fibrosis are usual. The former causes portal hypertension, the incidence of which is reflected in the weight of the spleen, which is usually from two to three times enlarged, the average of 66 records being 416 g. (Sheldon, 1935). In this case the histology of the spleen did not suggest portal hypertension, and the weight, 256 g., was only slightly above the normal range.

Absence of Haemofuscin.—Haemofuscin is a common pigment in haemochromatosis and fails to give the free iron reaction. Pigmentation of the heart, gastric mucosal glands, and adrenal cortex is uncommon in haemosiderosis. This was found to a moderate degree in the present case, and more in keeping with idiopathic haemochromatosis.

Discussion

Uncritical acceptance of cases of haemochromatosis after blood transfusions has led to a false impression of the prevalence of this condition. Dubin (1955) suggested that the criteria required for the diagnosis should be the same as those required for idiopathic haemochromatosis, namely, (*a*) true cirrhosis, with unmistakable pseudo lobulation resulting in portal hypertension and fibrocongestive splenomegaly : (*b*) pancreatic fibrosis ; and preferably (*c*) diabetes mellitus.

When these criteria are used the majority of cases reported are unacceptable as examples of a condition identical with haemochromatosis.

There remains a small group of cases in which a true cirrhosis of the liver and pancreatic fibrosis has accompanied haemosiderosis. These cases still differ in that the distribution of iron is mainly in reticulo-endothelial cells as opposed to its location primarily in epithelial tissues in haemochromatosis, and also in the absence of haemofuscin (Dubin, 1955).

Experimental evidence to support the contention that excessive iron deposits in transfusion siderosis are of themselves the primary cause of the hepatic and pancreatic fibrosis has not been forthcoming, despite the researches of Brown, Moore, Reynafarje, and Smith (1950), Cappell (1930), Polson (1933), and Wyatt and Howell (1953). Further doubt has been raised by the lack of correlation generally recognized between the amount of iron administered intravenously and the development of haemochromatosis, a presumption illustrated by the case reported by Chesner (1946). It is also remarkable that many tissues are free from damage both in haemochromatosis and haemosiderosis despite heavy deposition of iron, and massive pulmonary siderosis from inhalation of iron does not cause pulmonary fibrosis (Doig, 1950).

It must be accepted that cases will occur which are clinically indistinguishable from primary haemochromatosis, and in some few of these the diagnosis has withstood a careful necropsy and

critical histological examination of the tissues. It may be that the toxic action of iron is dependent on various factors such as the amount of iron administered (Finch, Hedsted, Kinney, Thomas, Rath, Haskins, Finch, and Fluharty, 1950; Morningstar, 1955), the route of its entry, or the duration of its presence in the tissues (Schwartz, 1956). Haemofuscin may not be important in considering the toxicity of iron, as this pigment is generally considered to be free of iron (Sheldon, 1935).

The following interesting problems remain unsolved:

(*a*) The toxicity to the tissues of iron compounds; (*b*) the cause of hepatic cirrhosis and pancreatic fibrosis in idiopathic haemochromatosis; (*c*) the incidence of haemochromatosis after blood transfusions.

Further study may be helped by the careful reporting of critically examined cases.

Summary

A case of transfusional haemosiderosis simulating haemochromatosis is reported.

The points of difference between this case and idiopathic haemochromatosis are enumerated, and various similarities noted.

The evidence for and against the toxicity of iron in the tissues is presented.

The literature on the subject is briefly reviewed.

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J. clin. Path. (1958), **11**, 133.

CEREBRAL THROMBOANGIITIS OBLITERANS

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A stroke due to vascular occlusion in a young person can raise many diagnostic and prognostic problems. In the following case report hemiplegia of sudden onset occurred in a young man followed by partial recovery without a definite diagnosis being made, despite full investigation. Further progression of the disease leading to death two years later enabled a complete pathological investigation to be undertaken.

Case Report

A 26-year-old labourer was struck on the right side of the forehead on April 30, 1952, by a 5-gallon can while loading it on to a wagon. He sustained a small cut, 6 mm. in length, over the right eye and was able to continue at his work. About two hours later he had an epileptiform fit in which his speech became slurred and the left side of his body became weak. He had been in good health previously. There was a history of convulsions in early childhood, an injury to the left eye when 9 years old, and osteomyelitis of the jaw when 13 years old. On admission to this hospital a week later he was found to have a profound left hemiplegia and hemianaesthesia which had remained unchanged since the fit. His blood pressure, blood count, and cerebrospinal fluid were normal. The Wassermann reaction was negative both in the blood and the cerebrospinal fluid. A right-sided percutaneous angiogram showed normal filling of the middle and anterior cerebral vessels. Air ventriculography outlined small narrowed ventricles, slightly displaced to the left of the midline. An E.E.G. showed a high-amplitude slow-wave focus in the right fronto-temporal region, consisting of 2 to 4 c./sec. waves. These findings were interpreted as being due to right-sided cerebral oedema in relation to an infarct or possibly an intracerebral blood clot.

When the air study was repeated three weeks later, the ventricles appeared in their normal position, but the right ventricle had become dilated, thus supporting the diagnosis of infarction. The E.E.G. at this time showed improvement on the previous record, with right-sided low-voltage slow waves. When repeated three months later the E.E.G. was normal. The left hemiplegia persisted with marked spasticity of the leg and flexion deformity of the hand. The only movement in the left upper limb was a slight elevation at the shoulder joint.

About 18 months after the first attack he had a further series of left-sided fits, despite being on anticonvulsant drugs. Several E.E.G. tracings at this time were normal. After a severe fit on February 9, 1954, he remained incontinent and unable to speak or swallow. He now presented the picture of pseudo bulbar palsy, with bilateral increased reflexes and extensor plantar responses. He showed gross emotional lability with frequent episodes of depression and weeping. The E.E.G. showed irregular moderate amplitude 3 to 4 c./sec. activity in the left frontal region. A repeat air encephalogram two weeks later was normal. The cerebrospinal fluid and blood pressure were again normal on examination. He continued to have occasional fits and his condition deteriorated. He died on May 5, 1954, three months after developing pseudo bulbar palsy and two years after the onset of his illness.

Pathological Findings

At necropsy, performed 18 hours after death, no evidence of injury was found apart from an old corneal opacity of the left eye which had been present since childhood. The body was thin but not wasted. The larynx and trachea were healthy. Both lungs showed basal congestion. The heart was normal in size and configuration, with healthy muscle and valves. The alimentary system, liver, spleen, and endocrine organs appeared healthy. Both kidneys contained several small wedge-shaped scars in the cortex with normal intervening tissue. The renal arteries were healthy. Slight patchy atheroma was present in the descending aorta. Several arteries, including the coronary, carotids with internal and external branches, subclavian, axillary, internal and external iliac, and femoral, were examined on both sides. All were healthy and free from atheroma.

Central Nervous System.—No abnormality of the skull or dura was seen. The pia arachnoid was thickened over both hemispheres, maximally over the fronto-parietal regions, where it was discoloured golden brown by haemosiderin pigment. The convolutions surrounding the upper part of both Sylvian fissures were shrunken and depressed below the adjacent gyri as a result of cystic

softening (Figs. 1 and 2). The area affected was more extensive on the right side, extending deeply to involve the claustrum, external capsule, and putamen, whereas only the insular cortex and underlying white matter were included in the softening on the left side (Fig. 3). Similar but much smaller areas of old softening were present on the under surface of both occipital lobes. These had destroyed the fusiform gyri, more extensively on the right side than the left. The vessels of the circle of Willis were healthy and free from atheroma. On dissecting out the three major vessels, the anterior and posterior cerebral arteries appeared normal. The middle cerebral arteries were healthy at their origins. Distal to their first main branches, both vessels were small, white, and thread-like without an obvious lumen. The brain-stem and cerebellum were normal.

Histological Examination

The infarct in the right insula region has progressed to cyst formation with the wall of the cyst formed by the internal capsule and globus pallidus. The contents of the cyst have disappeared, but scattered histiocytes and proliferated capillary vessels are present in its margin. There is dense fibrillary gliosis around the affected area, and vessels in the surrounding tissue are cuffed with lymphocytes and a few histiocytes. Many of these cells are present in the overlying thickened pia arachnoid.

FIG. 1.—Right lateral aspect of brain showing extensive area of old softening around the Sylvian fissure.

FIG. 2.—Left lateral aspect of brain showing much smaller area of softening.

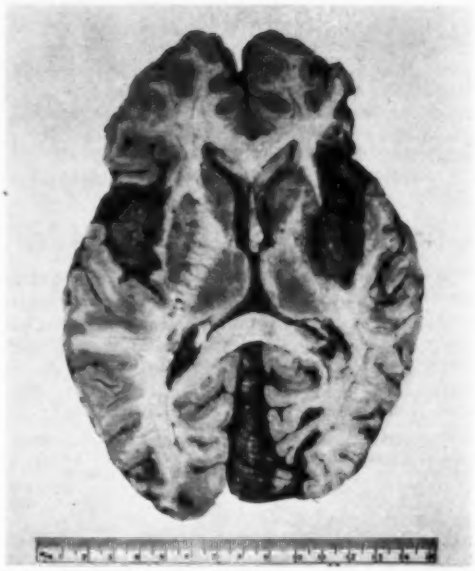

FIG. 3.—Bilateral softenings of the insula region more extensive on the right side.

A small area of cystic softening, similar in age and appearance, is present on the under surface of the right occipital lobe. This involves the cortex and underlying white matter of the fusiform gyrus with dense gliosis of the surrounding tissue and of the molecular layer of the affected cortex.

The infarcts in the left insula region and on the under surface of the left occipital lobe are much less extensive and of more recent origin. The affected areas are filled with numerous histiocytes and lymphocytes, loosely arranged between proliferated capillary vessels. There is gliosis of the overlying molecular layer of the affected cortex and around the softened areas, but this is much less dense than on the opposite side.

In addition to these large necrotic areas there are scattered small glial scars in the cortex of the right and left superior temporal gyri and in both occipital lobes. In these areas there is loss of nerve cells and dense gliosis.

FIG. 4.—Transverse section of the right middle cerebral artery distal to the origin of its first branch. Elastic Masson stain, × 35.

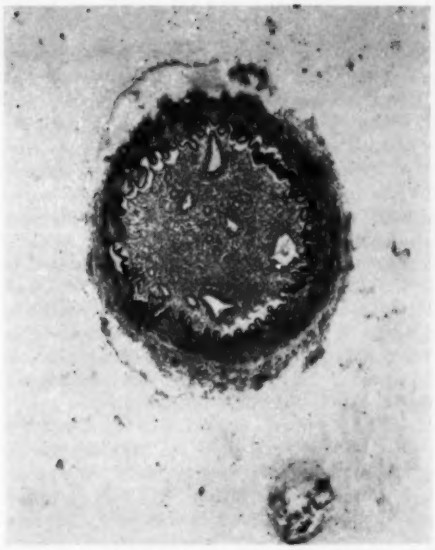

FIG. 5.—More distal part of the right middle cerebral artery. Elastic Masson stain, × 35.

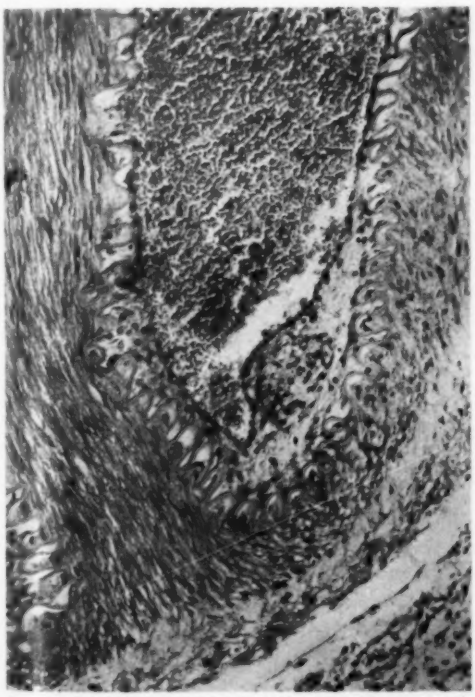

FIG. 6.—Proximal part of the left middle cerebral artery showing subendothelial connective tissue proliferation. Haematoxylin and eosin, × 120.

FIG. 7.—Splitting of internal elastic lamina. Proximal part of left middle cerebral artery. Elastic Masson stain, × 89.

In the brain-stem there is descending degeneration of the cortico-spinal tracts, worse on the right side than on the left.

The right middle cerebral artery beyond the origin of its first branch (Fig. 4) shows recanalized occlusive thrombus, the central mass consisting of swollen fibroblasts amongst which are a few small round cells and occasional histiocytes containing haemosiderin pigment, and including several small, separate, endothelial-lined channels, one of which is occluded with recent fibrin thrombus. The internal elastic lamina is intact, except in one place where it shows reduplication. The media and adventitia appear healthy. These changes extend about 1 cm. along the vessel where the endothelial-lined channels become smaller and fewer (Fig. 5). More distally the lumen of the artery is completely blocked by proliferation of the subendothelial connective tissue of the intima.

The left middle cerebral artery shows similar changes distal to its first branches. The proliferated connective tissue is much more cellular than on the right side with less dense collagen. The most proximal segment of the artery shows a small localized area of subendothelial proliferation (Fig. 6), with fraying and reduplication of the internal elastic lamina (Fig. 7).

The posterior cerebral arteries are healthy, but, over the infarcted areas in the occipital lobe, the terminal branches show severe endarteritis with narrowed lumina (Fig. 8).

Several wedge-shaped scars of dense fibrosis in which fibrosed glomeruli can be recognized are present in the kidneys. No thrombosed vessels are seen and the arteries sectioned appear normal.

The vessels in both pectoral muscles show no evidence of periarteritis nodosa.

FIG. 8.—Endarteritis of smaller branches of left posterior cerebral artery. Haematoxylin and eosin, × 115.

Discussion

Before this case can be accepted as one of cerebral thromboangiitis obliterans without involvement of the peripheral limb vessels, it is necessary to rule out the various other possible causes of cerebrovascular occlusion. There was no previous history of rheumatic fever and the heart showed no evidence of rheumatic disease. The blood pressure was within normal limits on every occasion it was recorded. Although recanalized impacted emboli might give a similar appearance in the cerebral vessels, no possible source of embolic origin for the infarcts was found, while infarction of the same age, as assessed histologically, in the territory supplied by both middle and posterior cerebral arteries does not support this mechanism. Emboli have occurred from the site of the puncture after percutaneous angiography (Crawford, 1956), but this investigation was undertaken after the onset of hemiplegia on each occasion. The age of the patient is against arteriosclerosis, and no evidence of any generalized disease, such as diabetes, syphilis, or tuberculosis, was found. The trauma sustained before the onset of the first symptoms was relatively trivial and could not have damaged the cerebral vessels.

The microscopic changes in the middle cerebral arteries were confined to the intima with preservation of the internal elastic lamina and no inflammatory changes and necrosis, thus favouring thromboangiitis obliterans rather than polyarteritis. The pathological distinction in the later stages between thromboangiitis obliterans and polyarteritis nodosa may be difficult, as in the case reported by Treip and Porter (1957). If hypersensitivity should be the basis of both of these conditions (Llavero, 1948) overlapping of histological lesions may well be possible. Although most authorities regard the histology as distinct, Boyd, Ratcliffe, Jepson, and James (1949)

questioned the specificity of the pathological findings in thromboangiitis. The original contention of Buerger of the specificity of "purulent giant cell foci" has been denied by many workers, including Koyano (1922) and Krampf (1922). No such foci were found in the present case. No evidence of venous thrombosis was seen, but, as this occurs in only about 30% of cases of peripheral Buerger's disease (Brown and Henderson, 1927), it is not an essential accompaniment for the diagnosis. Thus, although endarteritis can be part of the pathological process in a number of well-defined conditions (McLetchie and Gillis, 1955), these have been considered and rejected. The opinion of Julitz (1953) that many of these cases are essentially Buerger's disease of widespread, unusual, or variable distribution is accepted.

Although the essential lesions in peripheral vessels were first described by von Winiwarter in 1879, it was Buerger (1908) who gave the disease the name by which it is generally known. In a subsequent study of these cases, Buerger (1924) described death in some from cerebral complications, but attributed these to arteriosclerosis. It was Cserna (1926) who first described the disease affecting cerebral vessels. Barron and Linenthal (1929) described two cases with hemiplegia and one with basilar thrombosis. Jäger (1932) showed that the vessels of many viscera, including heart, kidneys, stomach, pancreas, and brain, could be affected and stressed the diagnostic importance of the connective tissue filling the lumen of the thrombosed vessel (*Füllgewebe*). Bauer and Recht (1932) recorded a case with repeated cerebrovascular occlusions. Foerster and Guttmann (1933) made a clinical diagnosis of cerebral thromboangiitis obliterans without reporting on the pathology. Spatz (1935) gave a clear description of the disease involving peripheral and cerebral vessels, but this case is now thought to be one of recurrent emboli.

In the last 20 years many reports have appeared which conclusively establish the existence of the disease in cerebral vessels (Minkowski, 1947–48; Llavero, 1948), but there are relatively few histopathological studies concerning the brain changes and confirming the diagnosis. Sträussler, Friedmann, and Scheinker (1937) recorded the pathological details of a case. Hausner and Allen (1938) recorded 11 cases with cerebral symptoms out of 500 with thromboangiitis obliterans of the extremities (2.2%). In three of these cases the cerebral symptoms preceded those of peripheral vessel involvement by a varying time interval, the longest period being 14 years. Lindenberg (1939) recorded 22 cases of thromboangiitis obliterans of the brain, in 14 of which the arteries of the extremities were not involved, and Lindenberg and Spatz (1939) described the findings in 20 cases of the cerebral disease. Krayenbühl (1945) reported 25 cases of cerebral thromboangiitis and 11 cases were recorded by Davis and Perret (1947). In a recent survey of Silbert's series, Lippmann (1952) found nine cases of cerebral Buerger's disease in 1,700 cases with peripheral disease. Although Lippmann found over 250 cases of the cerebral disease recorded in the literature, only 30 were acceptable, and these, together with the nine taken from Silbert's series, were reviewed. In 12 of these 39 cases, cerebral symptoms developed before peripheral ones with a time interval of one to five years in eight cases and six to 10 years in three cases, while 20 years was the longest period recorded in one case.

Thus the statement of Klemperer (1943), that there must be a clinical history of involvement of the extremity before a diagnosis of thromboangiitis obliterans in visceral vessels can be accepted, is too rigid and narrow a conception of the disease in view of the well-authenticated cases of cerebral symptoms preceding peripheral ones by a long period of time. In the case described in this paper no peripheral symptoms or pathological changes were found, but as the time of survival was only two years from the onset of the illness it may well be that this was too short a time for more generalized disease to develop.

In many of the recorded cases thrombosis of the internal carotid artery is the principal or sole lesion and the diagnosis has been assumed, either from the absence of other known causes or from the presence of typical lesions in the cerebral arteries in the same case (Antoni, 1941). Stender (1936) found that the anterior and middle cerebral arteries were most often involved, especially the latter. Lippmann (1952) found the internal carotid, vertebral, middle, anterior, and posterior cerebral arteries involved in decreasing order of frequency. The majority of reported cases have been diagnosed at necropsy as in the case recorded here, although Sorgo (1939) and Sunder-Plassmann (1941) described the obliterated cerebral arteries in arteriograms. The angiogram in the present case was reported as normal and subsequent review of the films after the necropsy failed to show any abnormality. In view of the disease affecting the middle cerebral arteries beyond their first branches which were patent and healthy, the normal angiographic appearances can be understood.

Concerning the pathological findings, the gross appearance of the affected arteries resembled the

description given by Antoni (1941) and Lüers (1942). The microscopical appearance gave no indication of an acute inflammatory process as described by Buerger. No evidence of a primary necrobiotic stage with intramural haemorrhage in the subendothelial tissue between the intima and media was found as described by Scheinker (1944, 1945). The appearance is one of proliferation of endothelial cells of the intima and of the subintimal fibrous tissue corresponding with the second stage described by Scheinker and confirmed by Davis and Perret (1947). The presence of histiocytes filled with blood pigment in the swollen fibroblastic layer may be an indication of old haemorrhage at this site, but the haemosiderin could equally well have originated from degenerate red cells in a thrombus. The scanty lymphocytes were the only indication of a possible inflammatory basis for the condition.

Thus, although the findings in this case are far removed from the concept of the disease originally described by Buerger, they resemble those of a small but well-defined group of cases recorded in the literature and are characterized by typical obliterative arterial disease in the young adult. Even in young males with occlusion of limb vessels, the histological findings may vary considerably from those of classical Buerger's disease with the result that other names such as "non-specific obliterative angiitis" have been suggested (Pennock and Primas, 1956). Only the reporting of such cases with pathological correlation will clarify the limits of these groups and possibly indicate a more correct terminology.

Summary

A case of cerebral thromboangiitis obliterans is described in which no evidence of peripheral vascular involvement was found. The clinical features are correlated with the pathological findings and the differential diagnosis is discussed.

The cerebral manifestations of Buerger's disease reported in the literature are briefly reviewed, and the frequency with which these may precede symptoms in the limbs by varying periods of up to 20 years is stressed.

A diagnosis of the condition should be considered in any case of cerebral thrombosis in a young adult, where heart disease, arterial degeneration, and generalized systemic disease cannot be found, and demyelinating disease can be excluded.

My thanks are due to Mr. James Hardman, F.R.C.S., under whose care this case was admitted, for allowing full access to his notes and for much advice, and to Professor D. H. Collins for helpful criticism.

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PLEOMORPHIC SARCOMA OF THIGH WITH LONG SURVIVAL

BY

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In general it is fairly true to say that the prognosis in anaplastic malignant tumours of the soft tissues of the skeletal muscles is poor, but surprising exceptions do occasionally occur. The following two cases are examples of such exceptions and are reported because of their unexpected, long, and recurrence-free survival after local excision only. Both were rapidly growing, highly anaplastic tumours, of bizarre histological type, and thus were given a poor prognosis when first encountered.

Case Reports

Case 1.—In July, 1945, this 39-year-old turner complained of pain and an enlarging swelling, for seven weeks, in the antero-lateral aspect of the left thigh. The swelling was tender, fluctuant, and pulsatile, and the overlying skin was hot and reddened. The only other abnormal findings were a raised E.S.R. and slight pyrexia.

Blood only was obtained when the swelling was needled. At subsequent biopsy an unencapsulated, soft, and very vascular growth was found, with invasion of adjacent muscles. In August, 1945, the tumour was widely excised, most of the quadriceps apparatus and tensor fasciae latae being removed. Hypertrophy of residual muscles and recovery of function have been remarkable and no evidence of local or distant recurrence is apparent over 12 years after excision.

Case 2.—In May, 1948, a soft, ill-defined, and tender swelling was found in the right adductor magnus of this 44-year-old man. Pain, the presenting symptom, had been noticed for only two weeks, and at first the lump was thought to be a haematoma, there being no other abnormal feature. He was referred for physiotherapy but defaulted when the pain was quickly relieved.

Two months later he returned, the pain having recurred and the swelling now being double the previous size, firmer, and better defined. At biopsy a tumour was found lying deeply between the hamstring and the adductor groups of muscles in the upper third of the thigh. It was excised as widely as possible in July, 1948. The tumour measured $15 \times 7 \times 7$ cm. and was coarsely lobulated, soft, and vascular, with areas of haemorrhage and necrosis, and could be separated from the surrounding muscle bundles. While as much muscle as possible was removed, clearance on the deep surface was less than 1 cm. where the tumour had extended close to the femur.

Recovery of functional ability has been excellent and the patient remains free of tumour more than nine years after its removal.

Histology

The histological features of the two tumours were similar. They were highly cellular and largely formed of interlacing bundles of spindle cells, sometimes rather fibrillary, with eosinophilic, finely granular or vacuolated cytoplasm. The nuclei were plump, oval, and vesicular and usually contained a prominent nucleolus: two or more nuclei per cell, often arranged tandem fashion, were common. Reticulin and fibrous stromal tissue were relatively scant. In Case 2 many small round cells with scant cytoplasm were intimately mixed with the spindle cells and there were extensive areas of necrosis and haemorrhage.

A striking feature of both growths was the presence among the spindle cells of large numbers of bizarre and multinucleated giant cells (Fig. 1) which, in some areas, were so numerous as to form the predominant cell type. These giant cells generally ranged from 30 to 90 μ in diameter, but others were much larger and measured as much as 200 μ in diameter. Their cytoplasm was eosinophilic, with fine to coarse granularity, or, less commonly, vacuolation. The nuclei were rounded, of variable size and number, vesicular to hyperchromatic, and generally contained a prominent nucleolus. They were obviously enormously polyploid, but often the nuclear chromatin was fused into a single irregular mass.

Normal and aberrant multipolar mitotic forms were common in both the spindle cells and giant

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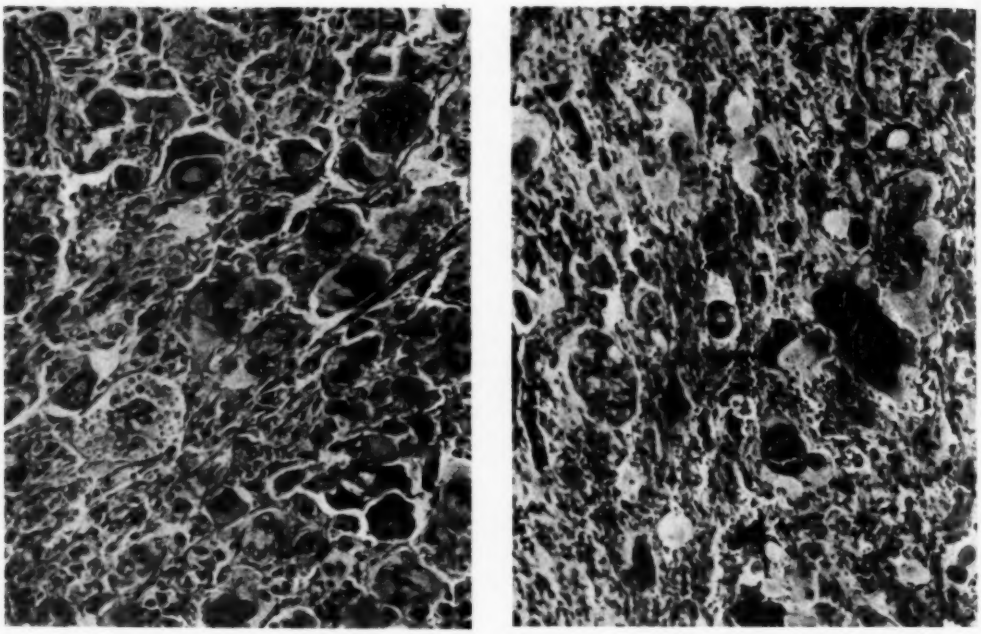

(a) (b)

FIG. 1.—(a) Case 1 and (b) Case 2: representative fields with many bizarre giant cells. In some areas the giant cells were even more numerous than here. Haematoxylin and eosin, × 110.

cells. While occasional large cells resembling myoblasts were present, racquet cells, strap cells, and sharply angulated cells were uncommon.

Both tumours showed extreme pleomorphism and morphologically they closely resembled rhabdomyosarcoma, but the failure to demonstrate cross striations prevented such a diagnosis being made with certainty.

Discussion

These cases are examples of anaplastic and pleomorphic neoplasms. The patients, while originally thought to have a poor prognosis, have unexpectedly survived and are remarkably well, with no recurrence, after long periods.

The failure to demonstrate cross striations leaves the precise nature of the growths in some doubt, but their general appearances are those of rhabdomyosarcoma. Some authors, such as Willis (1953) and Constance (1955), insist on the presence of cross striations before accepting any tumour as of striped muscle origin and tend to place pleomorphic growths such as these in the fibrosarcoma group. It is of interest, however, that in a case described by Lendrum (1947) no striations were demonstrated in the primary diaphragmatic tumour but they were clearly seen in the metastases.

Others do not require such rigid criteria for the diagnosis of rhabdomyosarcoma. Purdy Stout (1946, 1948, 1953), for example, excludes the possibility of this type of growth being a fibrosarcoma and believes that the presence of many bizarre giant cells indicates a derivation from other specialized cells such as rhabdomyoblasts. His series of 121 cases of rhabdomyosarcoma of skeletal muscle (1946) included examples showing no more precise features of striped muscle origin than do the two in question.

Whatever name is applied to these anaplastic growths, be it rhabdomyosarcoma, fibrosarcoma, or merely pleomorphic sarcoma, there is general agreement that the anaplasia and the bizarre giant cells are indicative of a high degree of malignancy, with a poor prognosis.

Nevertheless, in a few instances, long survival, even with persisting tumour, is recorded (Stout, 1946). Certainly it does seem that this type of

growth, with many giant cells, does sometimes fare rather better than those in which the predominant cells are small and rounded, as in some rhabdomyosarcomata. It may well be that the latter cell type is the more primitive, that it is the one which is more easily disseminated, and that it is from it that the giant cells are differentiated in the new implantation sites (Cappell and Montgomery, 1937; Cappell, 1948).

In any event, while a guarded prognosis must be given in pleomorphic tumours such as these, it is well to remember that long survival is sometimes possible, even without amputation, provided that surgical excision is adequate.

Summary

Two examples of pleomorphic and anaplastic tumours of the soft tissues of the thigh are described. While no cross striations were present they resembled rhabdomyosarcoma morphologically. The striking feature of each was the presence of numerous large and bizarre multinucleated giant cells.

Each patient was originally given a very poor prognosis, but they have survived, free of recurrence, for 12 and nine years respectively after local excision only.

We are grateful to Mr. R. Barnes and to Mr. R. A. Jamieson for the clinical details of these patients.

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HAEMANGIO-ENDOTHELIOMA OF THE SPLEEN AND LIVER

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With the exception of the reticuloses, primary malignant tumours arising in the spleen are rare, but from time to time examples have been reported which appear to be angioblastic in character and in some cases seem to cause metastases in the liver and elsewhere (Langhans, 1879; Theile, 1904; Jores, 1908; Dowd, 1915; Wright, 1928; Schlopsnies, 1929; Paine, 1931; De Navasquez, 1936; Wright, 1949; Abrahams and Hughes, 1952).

Although most authorities are agreed that malignant angioblastic tumours do occasionally occur, there is considerable doubt as to whether the tumours which appear to have arisen in the spleen are examples of true angioblastic malignant tumours or whether they represent multifocal systemic lesions (Willis, 1948). The case to be described appears to fall into this group and is reported because of its unusual interest, especially in regard to the age of the patient, and in the hope that eventually a true evaluation of these tumours may be forthcoming.

Case Report

The patient was an obese woman of 82 years who for some time previously had been suffering from degenerative cardiac disease with hypertension. For 18 months she had complained of constant left abdominal pain. A large mass was found in the left hypochondrium. At first this was thought to be a renal tumour but was later considered to be an enlarged spleen. A mild orthochromic anaemia was present. She remained in reasonably good health although showing progressive weakness and dyspnoea on exertion. She was finally sent to hospital in congestive heart failure, with ascites and dilated abdominal veins. She died shortly after admission.

Necropsy Report

The body was that of an obese woman showing cyanosis of the lips and extremities.

The brain and spinal cord showed no gross changes. There was a mild atheromatous degeneration of the cerebral vessels.

The heart was enlarged with marked hypertrophy of the left ventricle. The myocardium contained a patchy fibrosis. The valves were normal. The coronary arteries were narrowed by an extensive atheroma.

The lungs were congested and oedematous. No structural changes were apparent. The pleural cavities contained a small quantity of fluid.

A moderate amount of free fluid was present in the peritoneal cavity. The spleen was enlarged, measuring 18 cm. × 23 cm. × 13 cm. and weighing 490 g. It was oval and the surface smooth. The capsule was closely bound to the left cupola of the diaphragm. The cut surface was mottled with areas of spongy vascular tissue interspersed with hard white trabeculated tissue. There were a few small scattered cysts filled with blood. Anatomically the splenic and hepatic vessels were normal, but the splenic artery was enlarged and the vein dilated.

The liver showed a fine cirrhosis. Large numbers of vascular tumours were scattered throughout the left lobe, but there were no tumours in the right lobe. Those on the surface did not protrude above it. The tumours varied in size from 2 to 3 mm. up to 3 cm.

The other abdominal organs appeared normal and no enlarged lymph nodes were to be found. No abnormality was found in the marrow and no bone deposits were detected.

Histology

Spleen.—The splenic tissue is almost entirely replaced by angiomatous tissue and hyalinized fibrous tissue. Only a few degenerate Malpighian corpuscles are to be found, and these appear to be largely replaced by angioblastic tissue which is capillary in some areas whereas in others a cavernous structure is present. The vessels continuously branch and anastomose and form a tangled mass, but in places there is no canalization and this gives an appearance of new vessels being formed. There is, however, no sign of active growth. In other areas the tumour is more cellular and sheets of endothelial cells are present without any apparent vascular formation (Fig. 1). Here the cell outlines are indistinct, but the size appears to be regular and, apart from the nuclei being large and hyperchromic, there are few of the accepted criteria of malignancy and mitoses are very rare. The capsule of the spleen is normal in appearance and shows no sign of invasion.

Liver.—Sections of the nodules in the liver show a similar pattern to that seen in the vascular areas of

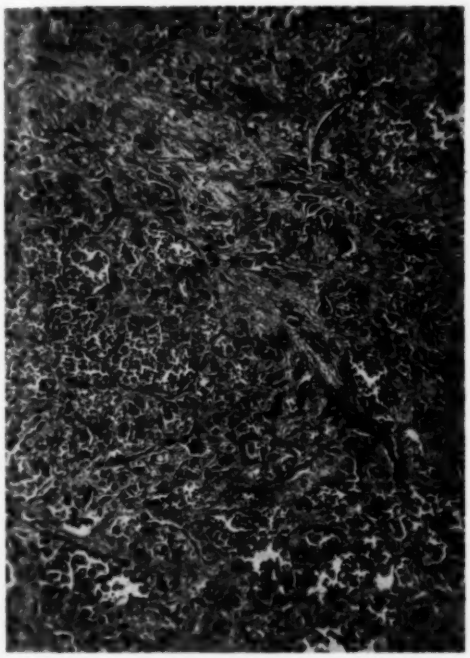

FIG. 1.—Section of spleen showing sheets of endothelial cells and open vascular channels. (× 95.)

the spleen, although there is more variation in the size and shape of the cells. Once again mitoses are almost completely absent although the endothelial cells give the appearance of infiltrating between the liver cells (Fig. 2). Careful examination, however, reveals that these cells merge into the normal capillary pattern of the lobule and do not seem to be spreading up the capillary channels nor do they show active growth, and indeed in many parts there are well-formed capillaries (Fig. 3). The liver cells are being destroyed by pressure.

Discussion

Although proliferative angiomatous lesions are extremely common, there is doubt as to whether they should be considered as true tumours or congenital vascular malformations. Harvey, Dawson, and Innes (1940) have stated that the term "angioma" should include all pathological vascular proliferations which are not regenerative, inflammatory, or mechanical disturbances of the normal circulatory arrangements and that there is little point in stressing the difference between congenital malformations and true tumour. It is admittedly sometimes extremely difficult, if not at present impossible, to differentiate between some malformations and tumour. Nevertheless the attempt is worth while, for it is by striving to solve such problems that advances are made in understanding tumour genesis.

If true angioblastic tumours do occur it seems reasonable to assume that a more malignant form might exist than the simple, ubiquitous angioma, but a review of the literature reveals that such aggressive lesions are extremely rare, and Willis (1948 and 1952) is reluctant to admit the existence of malignant angioblastic neoplasms, preferring to consider them as merely vasoformative variants of mesenchymomas, and maintaining that the majority of reported cases are examples of multifocal systemic lesions. Winston Evans (1956) seems to agree with this view but at the same time has illustrated the author's case as an example of the so-called malignant haemangio-endothelioma.

The case reported here, which appears to resemble in many respects that described by De Navasquez (1936), contains many features which at first sight seem to indicate malignant propensities. The formation of anastomosing, imperfect

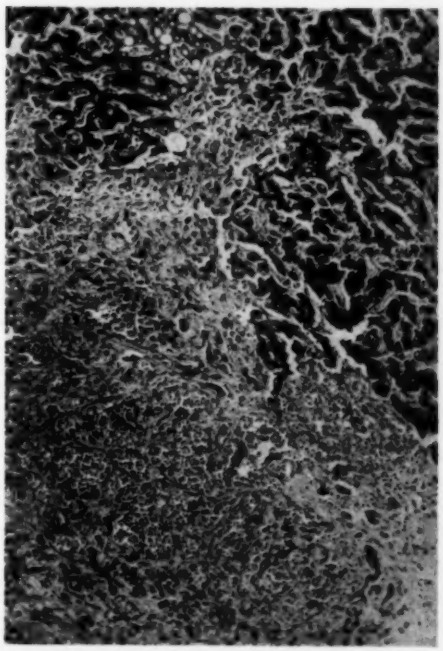

FIG. 2.—Section of liver, showing well-formed vascular channels in the tumour. At the edge the endothelial cells show apparent invasion with some destruction of the liver cells. (× 95.)

FIG. 3.—Section of liver showing open vascular channels destroying the liver cells by pressure. (× 95.)

blood channels by rounded endothelial cells with hyperchromic nuclei, and the filling of the vascular lumen with heaped-up cells, agree closely with the criteria of malignancy in these tumours as laid down by Stout (1943). It is also tempting to assume that the nodules in the liver, showing an apparent infiltration, are secondary metastatic growths from the primary growth in the spleen.

There are, however, many features which argue against a malignant interpretation. In spite of these histological characters, the cytology in the splenic tumour shows no evidence of rapid, high proliferative potentiality, and for two years the size of the spleen appeared to be constant. It might be that progressive fibrosis counteracted the proliferation of the cells causing their regression and conversion to fibrous tissue, a well-recognized feature of angiomata. The cells of the lesion also exhibit few or no mitoses and no evidence of active capillary sprouting as described by Stout (1943). In addition there is no suggestion of invasion of the splenic capsule in spite of the fact that the whole of the spleen appears to be converted into vascular tissue. If the tumour had been found to be confined to the spleen then there would be no reason to consider it to be malignant, although a type of tumour is recognized in which the primary growth is well differentiated but a recurrence is subsequently found to be truly malignant in character and disposition (Hill and Stout, 1942).

Examination of the nodules in the liver shows that here again there is no evidence of active growth ; nevertheless there is an apparent invasion of the liver with destruction of the cells. Careful scrutiny, however, reveals that this apparent invasion is only occurring in one or two areas around the nodules. The endothelial cells in these parts are well differentiated and indeed in some cases show perfect capillary formation. It would seem that these areas are the " feeding " vessels of the angioma, and the destruction of the liver cells is due to compression owing to a recent opening up of more channels. Willis (1948) has pointed out how poorly demarcated are hamartomas and how their mingling with normal tissues gives a false appearance of infiltrative activity. It is difficult to conceive that a tissue, so capable of rapid proliferation as is vascular endothelium, should show so little sign of proliferative growth.

Another feature of considerable interest is the occurrence of cirrhosis. This feature was also present in the case described by Ogilvie and Mackenzie (1936). They believed that their tumour arose in a previously cirrhotic liver. The association of cirrhosis with hepatic endothelial tumour has been regarded as evidence favouring the theory of their origin by a process of regeneration of endothelial channels (Evans, 1956). If this were so it would seem possible that the same process could occur in a fibrotic spleen. The vascular structure of the spleen and the left lobe of the liver are closely bound together and it might be expected that any stimulus affecting the one would also affect the other. It is noteworthy that in spite of the large size of the splenic growth and multiple hepatic angiomatous nodules there was no evidence of angiomatous tumours in the lungs or elsewhere nor could there be found any evidence of haemopoiesis as described by Paine (1931) and Hastings-James (1949).

It is easier to conceive of a vascular defect giving rise to an angiomatous malformation of the spleen and, to a lesser extent, the left lobe of the liver than tumours of the kind described metastasizing. Such a defect might remain indolent, growing slowly throughout life until because of the onset of a congestive heart failure the enlarged spleen was noticed during an examination. This congestive failure, together with a slowly advancing

cirrhosis of the liver and fibrosis of the spleen, might be the cause of the opening of the vascular channels with compression of the liver cells, so deforming them as to give an appearance suggestive of malignant invasion.

Such an explanation, denying the malignancy of this tumour, is more in keeping with present ideas of the mutability of cells. If the endothelial cells should undergo neoplastic change they are more likely to give rise to a mesenchymoma or possibly a fibrosarcoma, perhaps with vasoformative tendencies, and not to reasonably well-formed angiomatous tissue.

Summary

A case of haemangio-endothelioma involving the spleen and liver is described and illustrated. Reasons are given for believing that this is a systemic malformation rather than a true tumour.

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A CASE OF WEGENER'S GRANULOMATOSIS

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In 1931 Klinger described a case of polyarteritis nodosa with an unusual localization of the lesions in the nasal septum, lungs, spleen, and kidneys. Wegener (1937, 1939) reported three more similar cases as a new disease, stressing its granulomatous aspect. Since that time, examples of this condition have appeared in the literature under a confusing variety of different names, but recent authors have preferred the term "Wegener's granulomatosis," and the present state of our knowledge of this subject is summarized by Fahey, Leonard, Churg, and Godman (1954) and by Godman and Churg (1954). In two excellent papers they review the literature, describe seven new cases and clarify the diagnostic criteria of this disease. The present paper describes another case of Wegener's granulomatosis and discusses its relationship with some cases of Ceelen's haemosiderosis. The story of the case and its investigations will be more readily understood if it is appreciated that it presented as an intractable anaemia and was investigated and treated as such. Indeed, apart from haemoptysis, thought to originate from an ulcer of the nasal septum, symptoms referable to the respiratory system were extremely slight.

Case History

D. F. M., a butcher aged 40, first went to see his doctor in January, 1949, on account of "rheumatism." He had no fever but complained of painful, swollen joints which improved in a few days.

On February 28 he was sent to a chest clinic with a note from the doctor specifying cough, haemoptysis, and night sweats.

The clinic notes stated that cough had been present for several weeks, sputum, 1–2 oz., blood-stained for several days, pain in the left chest on and off. The appetite, bowels, kidneys, and bladder were normal. The fingers were not clubbed. The patient did not suffer from lassitude or loss of weight, but was in poor general condition. A few moist sounds were heard at the apex of the left lung, and a chest radiograph suggested right basal bronchiectasis.

A review of the radiograph showed a thickened right interlobular pleura and generally increased bronchovascular shadows with possibly diffuse fine fibrosis.

On March 23 the patient was sent to the chest clinic complaining of haemoptysis every morning after "a cold" at Christmas, 1948, and also of multiple septic skin lesions. On examination, ulcers on the nasal septum and right inferior turbinate and a punched ulcer on the palate immediately above the uvula were seen. The Wassermann reaction was negative. He was given potassium iodide, and on March 30 he had an "iodine" rash and herpes labialis. The Wassermann reaction was still negative. By April 13 the palate ulcer had healed, but septal bleeding continued. On May 17 a biopsy of the septal ulcer showed chronic inflammatory, nonspecific granulation tissue, with no evidence of tuberculosis or malignancy.

On June 5 urticaria was noted. Haemoglobin was 56%.

On June 13 he was sent to E.N.T. Department with letter: "A large slough separated from his right nostril to-day. During last fortnight he has had a septic throat. Recurrent herpes, angioneurotic oedema of face and urticaria. His general condition has not been too good and he has been in bed for some of the time."

An E.N.T. note on June 15 read: "His trouble is due to some obscure general condition. There is no enlargement of his liver or spleen." During his stay in hospital (three weeks) he ran a temperature of 99.4° F. on three evenings, otherwise he was practically apyrexial.

On October 6 his doctor wrote to the hospital: "M. had severe herpes around mouth and eyes. He came home from hospital about six weeks ago somewhat better, but has recently developed a septic toe—? ingrowing toe nail. About ten days ago a diffuse purpuric rash appeared, which is still present. He has had no pyrexia, but complains of rheumatic pains and general weakness."

On October 7 he attended hospital as an outpatient complaining of nasal catarrh and haemoptysis, which has been very slight since his operation. He did not feel well and had noticed herpes of the right eye, pains in both shoulders, slight cough, rash on the arms and legs, chiefly, for one month.

On examination, the right eye was seen to be injected. Purpura was everywhere, also septic spot. Nothing abnormal noted in heart, chest, and abdomen.

He was admitted to hospital on November 4. Hoarseness was getting worse.

On examination, he was rather pale, both eyes were infected, and he was very hoarse. He had a large ulcer on the left side of the palate, also septic spots and a purpuric eruption on the arms and legs. Blood pressure was 150/90 mm. Hg. Otherwise nothing abnormal was detected.

On November 7 R.B.C.s were 3.7 million and Hb 44%, and on November 9 two pints of blood (packed cells) were given.

By January 13, 1950, the ulcer on the palate was much larger, and also there was a sore on the scrotum.

On January 18 two pints of blood (packed cells) were given.

On January 20 he had an ulcer at base of the right index finger on the back of the hand. Two pints of blood were given on February 12.

The patient's condition declined steadily during the last month, and at the end, when he was unable to swallow on account of the extensive ulceration of the throat, his deterioration was rapid. He collapsed and was in coma for two days before he died on February 15, after being ill for 14 months. There was nothing in the clinical picture to suggest massive infarction of the lungs.

Radiographs taken on February 28 and November 7, 1949, showed a progressive condition (Figs. 1 and 2). In the first there is throughout both lungs a general increase in bronchovascular markings with patchy congestion, particularly marked at the right base and in the cardiophrenic angle. In the second film the condition is the same, but the general markings are finer and there are less mottled congestive areas. In a later film taken on January 10, 1950, there was a general fine striated appearance of both lungs, giving a ground-glass effect and suggesting a general fine fibrosis of both lungs.

Pathological Examinations

Haematology.—Between June, 1949, and February, 1950, 13 erythrocyte counts and haemoglobin estimations were performed. The counts varied between 2.9 and 4.2 million per c.mm. and the haemoglobin levels between 44 and 88% (Haldane), averaging approximately 50 to 60% with occasional rises after transfusion. The colour index was usually about 0.7 to 0.8 but rose to 1.0 a month before death. During the above period, 11 leucocyte counts were performed and the numbers ranged from 5,000 to 11,000 per c.mm. Polymorphs varied between 87 and 57%; eosinophils were noted on five occasions and varied between 1 and 2%. Three sternal marrow examinations were performed and the first two showed micronormoblastic erythropoiesis which was less severe in the third sample. All three tests showed hyperplastic marrow such as one finds with a secondary anaemia.

Reticulocytes.—5.0, 1.5, and 1.0% on December 14 and December 29, 1949, and January 10, 1950, respectively.

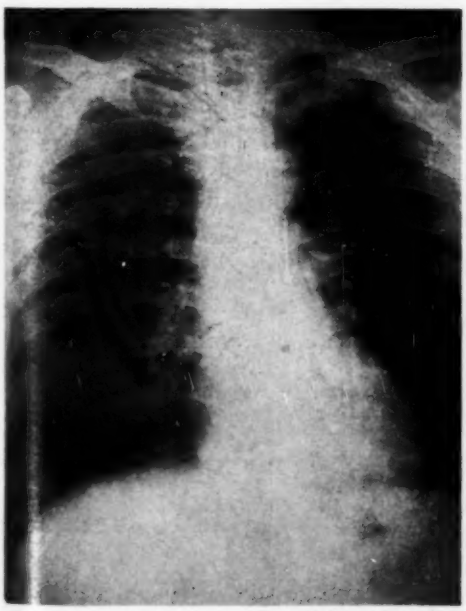

FIG. 1.—First radiograph of the chest on February 28, 1949.

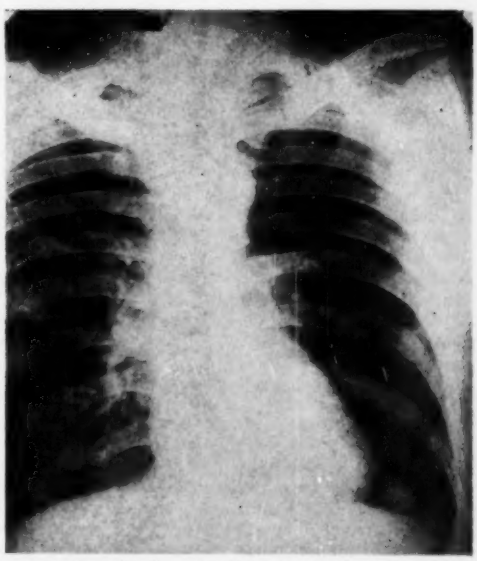

FIG. 2.—Second radiograph of the chest on November 7, 1949.

Platelets.—510,000, 500,000, and 480,000 per c.mm. on October 7 and November 7, 1949, and January 10, 1950, respectively.

Bleeding Time.—This was 3 minutes 15 seconds on July 15, 1949 (normal, 2 to 4 minutes).

Coagulation Time (Dale and Laidlaw).—This was 3 minutes on July 15, 1949 (normal, 1½ to 2 minutes).

The erythrocyte sedimentation rate (Westergren) was 68 mm. in one hour on July 26, 1949.

Erythrocyte Fragility.—Haemolysis began at 0.48% and was complete at 0.40% sodium chloride on February 3, 1950.

Serology.—Wassermann and Kahn tests were negative on March 23, March 30, and November 11, 1949.

Biochemistry.—A van den Bergh test was negative on January 17, 1950. The following results were obtained on February 3:

Alkaline phosphatase ..	10 units per 100 ml. (normal 3 to 10 units per 100 ml.)
Thymol turbidity ..	1 unit (normal 0 to 4 units)
Serum colloidal gold test	Negative
Van den Bergh test ..	Negative
Serum proteins	5·2 g. %
„ albumin	2·6 „
„ globulin	2·6 „ (A/G ratio 1 : 1).

Necropsy

Necropsy was performed on February 15, 1950, 18 hours after death.

External Examination.—The body is that of a pale, thin man of middle age. There are no purpuric haemorrhages, jaundice, or oedema. The dorsum of the right hand and the scrotum each show an ulcer 4 cm. in diameter. These are deep, penetrating ulcers with irregular, serpiginous edges, but they appear indolent and without surrounding induration, congestion, or inflammation.

Respiratory System.—The lungs present a striking appearance and bulge from the opened thorax. They are solid, voluminous, and fail to collapse when removed from the body. To the touch they are rubbery with firmer, ill-defined, rounded nodules scattered throughout their substance and measuring up to 2 cm. in diameter; they give the tactile impression of being condensations of fibrous tissue within a general diffuse pulmonary fibrosis. The lungs themselves are of a mottled red colour, varying from rust to dark blood red in places. There are occasional pale areas, notably at the right apex, but these are very scanty. The cut surface shows many haemorrhages and haematomata of varying ages, the older in some instances appearing to coincide with firm nodules. There is no evidence of pulmonary neoplasm or inflammation. Pleural adhesions and effusions are absent. The hilar lymph nodes are enlarged up to 2 cm. in diameter, but are quite soft. The trachea and bronchi contain blood-stained, frothy fluid. The palate is extensively ulcerated and covered with foul, necrotic debris.

Excretory System.—The kidneys are very pale and show moderately good demarcation of the cortex and medulla. They are of normal size and their capsules strip easily.

Cardiovascular System.—The heart is of normal size and without endocarditis or right ventricular hypertrophy.

The organs of the alimentary, reticulo-endothelial, and central nervous systems appear normal apart from extreme pallor.

Histology

Lungs.—The lungs show virtually no normal parenchyma and the following changes are found in varying proportions:

(1) Diffuse fibrosis with concentration of older collagen into ill-defined nodular areas (Fig. 3).

(2) There are many diffusely scattered small haemorrhages, each consisting of a group of involved alveoli. The haemorrhages appear to be of different ages, many of them recent. Some of the older haemorrhages seem to be related to the fibrous conglomerations.

(3) Heavy infiltration with siderophages, these being frequently concentrated into dense aggregations (Fig. 4). The amount of haemosiderin in these lungs is very large indeed.

(4) The minimal changes visible are collagenous thickening and diminished vascularity of the alveolar walls.

(5) An occasional group of alveoli is seen containing inflammatory exudate.

(6) Changes in the lung blood vessels consist of:

(*a*) Recent thrombosis of a few medium-sized vessels with patchy perivascular inflammation.

(*b*) Calcerosiderosis, distortion, and fragmentation of the elastica of many small vessels (Fig. 5).

(*c*) Acute arteritis of three small vessels with narrowing of the lumen, necrosis and fibrin deposition in the wall, destruction of the elastica, and perivascular leucocytic infiltration (Figs. 6 and 7). There is a zone of recent haemorrhage surrounding these vessels.

(7) The bronchial lymph nodes show numerous siderophages and carbon particles.

(8) The bronchi show thickening of the walls and sometimes notable cuffing with siderophages and inflammatory cells including lymphocytes, histiocytes, and plasma cells. The lumina of the bronchi may contain blood, desquamated epithelial cells, and a few siderophages.

(9) Destruction of elastic fibres in some alveolar walls and blood vessels.

Kidneys.—The kidneys show the following changes:

(1) About 20% of the glomeruli are abnormal, and these appear to be grouped together rather than scattered evenly throughout the renal parenchyma. The most common lesion is fibrosis of the tufts varying from slight collagenous thickening of the capillary membranes to dense scarring of the whole or part of the tuft (Fig. 8).

FIG. 3.—

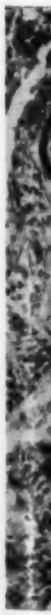

FIG. 4.—
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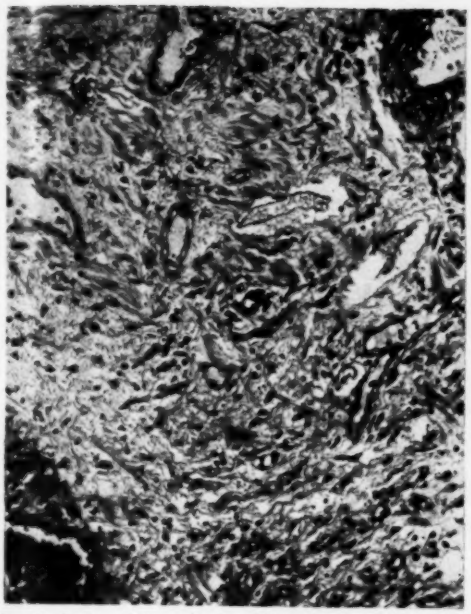

FIG. 3.—Section of lung showing diffuse fibrosis. Haematoxylin and eosin, × 94.

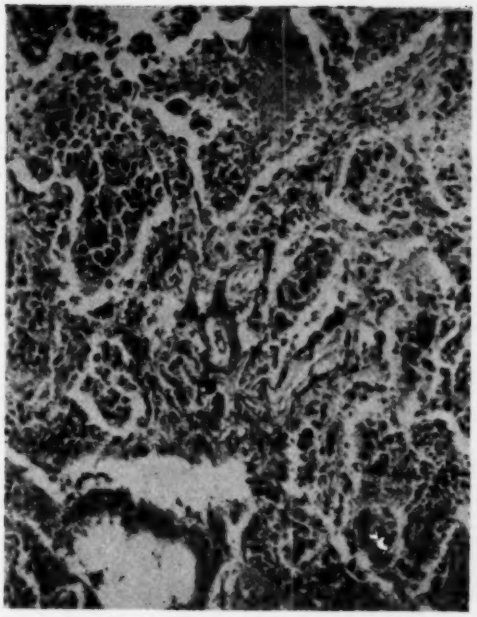

FIG. 4.—Section of lung showing large groups of siderophages. Haematoxylin and eosin, × 94.

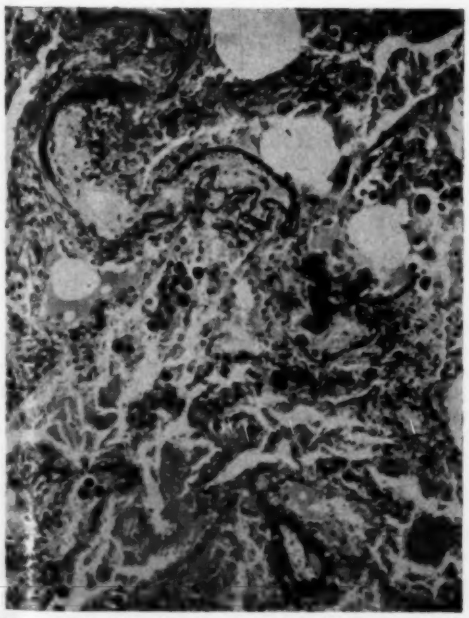

FIG. 5.—Section of lung showing calcero-siderosis of the elastica with distortion and fragmentation. Haematoxylin and eosin, × 94.

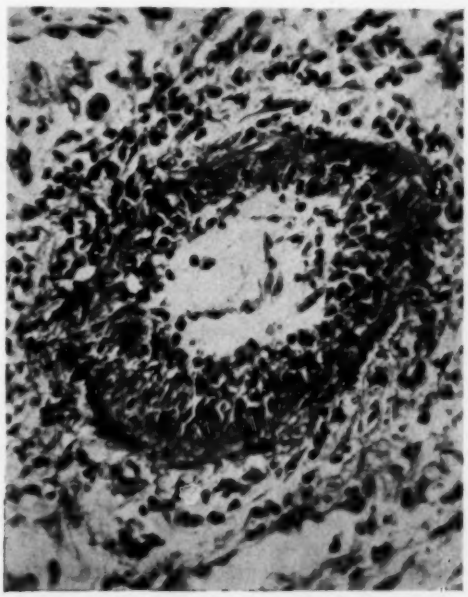

FIG. 6.—Section of lung showing necrotizing arteritis with destruction of elastica. Verhoeff's elastin with Masson's blue trichrome, × 240.

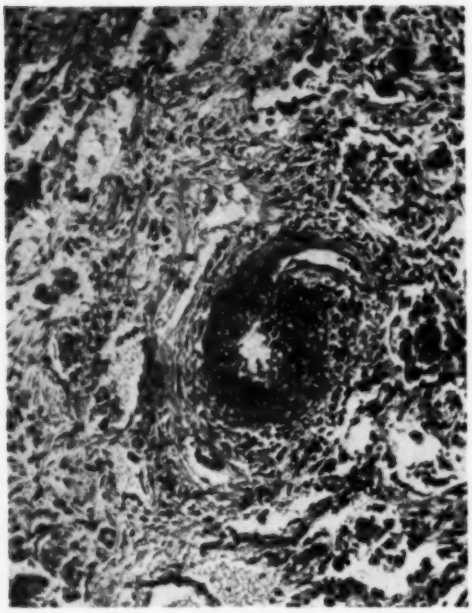

FIG. 7

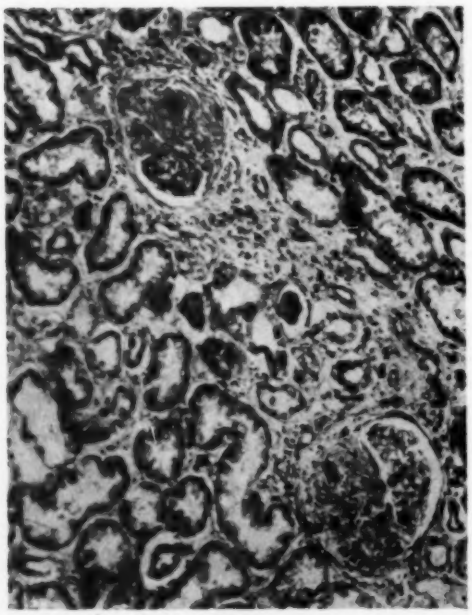

FIG. 8

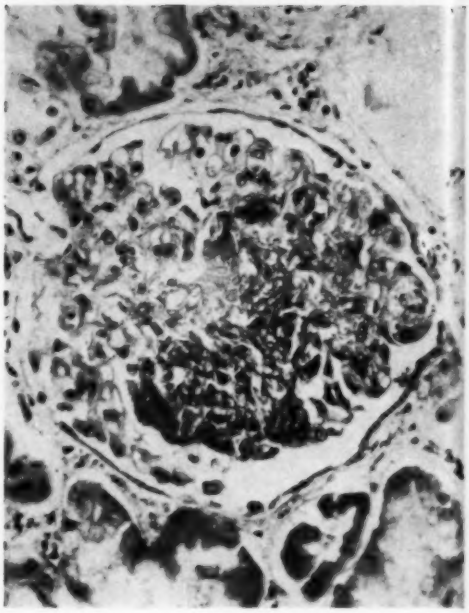

FIG. 9

(2) Some glomeruli show focal necrosis of part of the tuft. This may be associated with adhesive capsulitis (Figs. 9 and 10). Changes in the basement membrane of Bowman's capsule and epithelial proliferation within the capsule with demilune formation occur rarely.

(3) The tubules, especially the descending loops of Henle, show lipochrome aggregations in some epithelial cells. A few tubules contain red blood cells, hyaline and haematoxyphil material. Fatty change and desquamation of epithelial cells are not noted. There is no siderosis of the kidneys.

(4) The renal blood vessels appear normal apart from patchy fibroblastic thickening of the intima.

(5) The interstitial tissues show no oedema but some increase in collagen, particularly in the neighbourhood of diseased glomeruli. A few groups of inflammatory cells are present.

FIG. 7.—Section of lung showing a second vessel, near to that in Fig. 6 and similarly affected. Verhoeff's elastin with Masson's blue trichrome, × 94.

FIG. 8.—Section of kidney showing glomeruli, one with partial and the other almost complete fibrosis. Haematoxylin and eosin, × 94.

FIG. 9.—Section of kidney showing focal necrotizing glomerulitis. Haematoxylin and eosin, × 240.

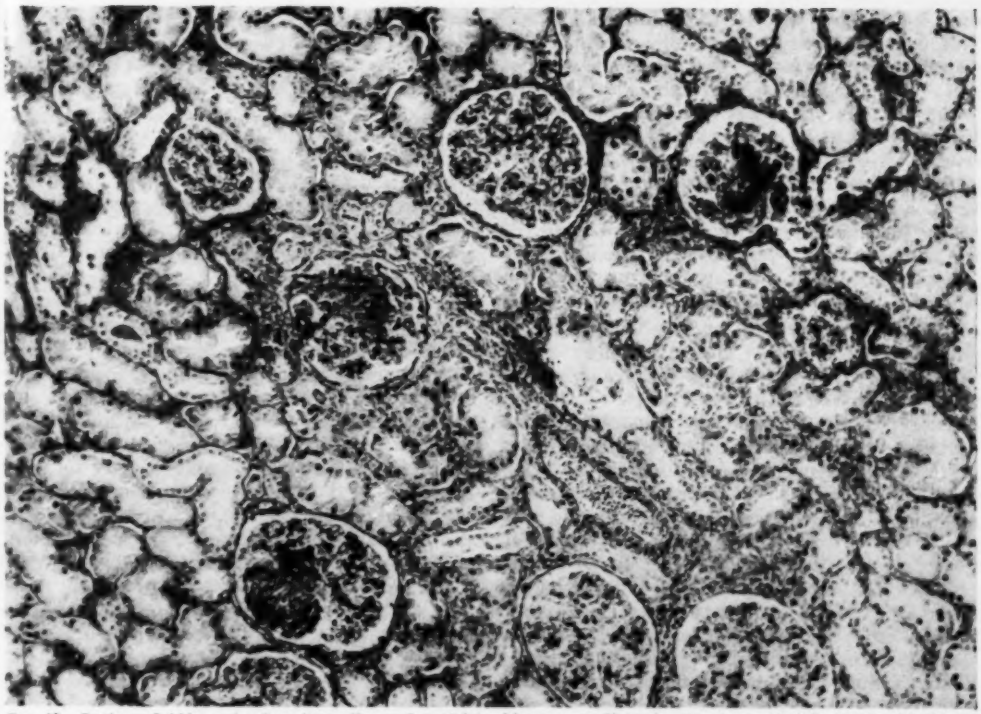

FIG. 10.—Section of kidney showing glomeruli, one (top right) with an acute fibrin deposit; a second (left centre) with fibrotic replacement; and a third (lower left) with a recent fibrin deposit at its centre. Picro-Mallory method, × 110.

Liver.—The liver shows extensive lipochrome infiltration of the parenchyma, particularly in the central cells, less in the portal region and least in the mid-zone. There is no siderosis or fibrosis of the liver, such as occurs in haemochromatosis. A microscopic area of vasculitis is noted.

Ulcers.—These, from the skin and palate, show extensive acute inflammatory changes with variable necrosis. Eosinophils are not numerous. Diphtheroids appear to be the predominant organism in the palatal ulcer. No necrotizing arteritis was noted.

Heart.—The heart shows atheroma and narrowing of the lumen of the interventricular branch of the left coronary artery.

Spleen.—The spleen shows a notable plasma-celled infiltration. The main structure is well preserved and no necrosis or arteritis was seen.

Discussion

The chief points of interest about this case are, first, its recognition as an example of Wegener's granulomatosis as distinct from polyarteritis nodosa, and secondly its relationship to Ceelen's haemosiderosis and the possibility that the latter may thereby be explained.

Syndrome of Nasal Granuloma, Pulmonary Angiitis, and Focal, Necrotizing Glomerulitis.—The coincidence of nasal granuloma and pulmonary arteritis was first reported by Klinger (1931), who described two cases of polyarteritis nodosa with what he called " an unusual localization of the predominant lesions in the spleen and lungs." Actually he was attempting to define a group of cases within the disease polyarteritis, but his group would nowadays be regarded as too ill-defined to merit recognition. The real interest in his paper lies in his first case, which began with disease of the maxillary antrum followed by ulceration of the nose and nasal septum; at necropsy the spleen and lungs presented arteritic changes and in the kidneys there was patchy or complete fibrosis or hyalinization of the glomeruli.

Wegener (1937) designated this disease as a " rhinogenic granulomatosis " with special involve-

ment of the arterial system and of the kidneys. This definition was elaborated by Godman and Churg (1954), who state that Wegener's granulomatosis is always characterized by the three following features: "(1) Necrotizing granulomatous lesions in the upper air passages or in the lower respiratory tract or both; (2) generalized focal necrotizing vasculitis, involving both arteries and veins, almost always in the lungs and more or less widely disseminated in other sites; (3) glomerulitis characterized by necrosis (and thrombosis) of loops or lobes of the capillary tuft, capsular adhesion and evolution as a glomerular lesion."

The chief difference between the diagnostic criteria of Godman and Churg (1954) and those of Wegener (1937, 1939) lies in the localization of the initial granuloma, and recent examples justify the extension of the definition to include all cases starting with a respiratory tract granuloma instead of restricting it to those of nasal origin. This disease, like other types of polyarteritis nodosa, presents so many variable features that only confusion results from regarding any one or two of these features as diagnostic. Wegener's original definition includes all that is constant and can be improved only by expanding the term "rhinogenous" to "respiratory" granulomatosis. Other lesions, which occur frequently but are not diagnostic, include pulmonary, renal, and splenic granulomatosis, which may or may not be associated with necrotizing arteritis. Fever, joint pains, and cutaneous lesions are often found, and the clinical picture is usually governed by the site of the initial granuloma. Leucocytosis, elevation of the erythrocyte sedimentation rate, and anaemia are the principal haematological findings. Widely differing degrees of severity may be encountered and most recorded examples are far more acute and severe than the present case. Its chronicity no doubt accounts for many of its unusual features including the lack of visceral granulomata, pyrexia, and leucocytosis and for the presence of a low-grade, intractable anaemia and the intense pulmonary haemosiderosis.

The main object in defining Wegener's granulomatosis is to differentiate a particular variety of polyarteritis nodosa distinguished by the presence of an initial respiratory tract granuloma, the involvement of only small vessels (veins as well as arteries), and the granulomatous evolution of the disease. The present case satisfies all these criteria, although the preponderance of haemosiderotic changes in the lungs and the cutaneous position of the secondary granulomata are atypical features. The type of vascular lesion in the viscera, including the lungs, and the characteristic focal lesions in the glomeruli, clearly resemble drug-induced vascular disease and the other hypergic affections of the blood vessels ("hypersensitivity angiitis" of Zeek, Smith, and Weeter (1948), "hyperergic fibrinous vasculosis" of Lendrum (1956)) far more than classical polyarteritis nodosa. So close is the similarity that it seems reasonable to regard the upper respiratory tract granuloma as the source of an allergen to which all the other manifestations can be attributed.

A tentative diagnosis of Wegener's granulomatosis may often be made on the clinical history of nasal or paranasal ulceration followed by signs and symptoms of polyarteritis nodosa, but in the end the proof is histological, depending on the demonstration of pulmonary, renal, and possibly visceral vasculitis. It must be realized that these lesions are essentially focal and may be very scanty. This is probably responsible for much of the confusion in the literature concerning cases such as that of Alexander (1954) where an arteritic lesion in the viscera can only be presumed from the gross description.

In the past some writers have adopted a quite different pathological pattern to characterize these cases, and, coupled with the use of over a dozen different titles, this has led to indescribable chaos in the literature. The two terms causing most confusion have been "giant-cell granuloma" and "malignant granuloma" of the nose. The former was used by Howells and Friedmann (1950) and by Seidelin and Willcox (1954) to describe undoubted cases of Wegener's granulomatosis. The epithet "giant-cell" is particularly undesirable, because the presence or absence of these cells in the lesions has little diagnostic value. Their presence may represent a reactive idiosyncrasy of the patient, similar to the capricious eosinophilia of some cases of polyarteritis nodosa, rather than a diagnostic characteristic of the disease. The journals of otology and laryngology contain a number of these cases under a variety of titles, the commonest of which are "malignant granuloma" (Woods, 1921) and "lethal granuloma" (Stewart, 1933). Their relationship to Wegener's granulomatosis must remain obscure, because the early workers were so preoccupied with the unfruitful study of the initial respiratory tract granuloma that no search appears to have been made for visceral arteritic lesions, even in those cases subject to necropsy. Some cases of lethal granuloma, such as that of Alexander (1954), may well be examples of Wegener's granu-

lomatosis; others almost certainly are not, and these include the case of McBride (1897) and the first two cases presented by Williams (1949), who made an extensive but unsuccessful search for visceral arteritic lesions. Moreover, Stratton, Price, and Skelton (1953) failed to find vasculitis in necropsy material from four cases of malignant granuloma, and Woodburn and Harris (1951) found evidence of polyarteritis nodosa in only one of six cases of lethal ulceration of the nose and face.

The number of cases collected from the literature by Fahey *et al.* (1954) and Godman and Churg (1954) is 22, to which they add a further seven of their own. Details of 11 more examples of Wegener's granulomatosis, together with the present case, are given in Table I, and this now brings the total up to 41. In addition mention may be made of a further intriguing case presented at a clinico-pathological conference (1955) and pertinent to both sections of this discussion. The patient, a girl of 16 years of age, died after an illness lasting eight months and starting with a bloody discharge from the left nostril. The necropsy findings included polyarteritis of the pancreas, healed focal embolic nephritis, and diffuse haemosiderosis of the lungs. Although the details are incomplete, it is interesting to note the comment that the necropsy findings in the lungs are best interpreted as "an incidental early case of haemosiderosis."

Some of the cases in the literature are inconclusive; for example no glomerulitis was demonstrated in Fienberg's case (1953) or in the first case of Godman and Churg (1954), and the renal lesions described by Klinger (1931) are not characteristic.

Pulmonary Arteritis and Ceelen's Haemosiderosis.—The condition of pulmonary haemosiderosis has been recognized by Lendrum (1950) as occurring in three forms, one associated with mitral stenosis, one with prolonged left ventricular failure, and the third, the type described by Ceelen (1931), which is a disease mainly of young people and quite unrelated to cardiac disease. This latter type under the title "idiopathic pulmonary haemosiderosis (essential brown induration of the lung)" was reviewed by Wyllie, Sheldon, Bodian, and Barlow (1948), who add seven new cases including a man of 38 years of age. Much of their description of the histopathology of the lungs could be applied exactly to the present case, but they attribute the disease to "an increase of reticulin, collagen and muscle and a decrease of elastic fibres in the pulmonary interstitial tissue, leading to a lack of distensibility of the lungs with consequent peripheral stasis in the capillary bed. This is followed by haemorrhage by diapedesis and deposition of haemosiderin." The histological changes described by Wyllie *et al.* (1948) are regarded by Lendrum, Scott, and Park (1950) as identical with those they themselves saw not only in one example of Ceelen's disease (since reported by Hutchison, 1954) but also in numerous examples of cardiac haemosiderosis; they considered that the incrustation and fractures of the elastic tissue were all secondary to the damaging effect of a locally high concentration of iron. These same authors have broadened the concept of Ceelen's disease by the inclusion of the cases described by Kockel (1899), by Sylla (1929), and by Engel and Newns (1939).

The more recent reports on Ceelen's disease include one by Hirrle (1952) with a brief review; he believes that a congenital hypoplasia of the elastic tissue is the most likely cause of the pulmonary haemorrhages. Defective vasomotor control of the lung capillaries followed by diapedesis

TABLE I

SUMMARY OF FINDINGS IN CASES STARTING WITH RESPIRATORY TRACT GRANULOMA FOLLOWED BY POLYARTERITIS

	Date	Author	Sex	Age	Duration of Illness (Months)	Site of Initial Granuloma	Organs Showing Arteritis
1	1936	Rössle	M	44	Not stated	Middle ear and nose	Lungs, spleen, kidneys, liver
2	1949	Williams	M	21	9	Nose, antrum, and ears	,, ,, ,, ,,
3	1951	Woodburn and Harris	M	31	9	Nose	,, kidneys, not spleen
4	1953	Ahlström *et al.* ..	M	70	2	Lung	,, spleen, and kidneys
5	1953	,, ,, ,, ..	F	41	14	Maxillary antrum	,, and kidneys, ? spleen*
6	1954	McCallum	M	31	2	Nasal septum	,, kidneys, spleen
7	1954	Seidelin and Willcox	M	55	14	,, ,,	Kidneys, spleen, lungs not described
8	1954	,, ,, ,, ..	M	54	18	,, ,,	Lungs, kidneys, spleen
9	1954	,, ,, ,, ..	F	55	8	Middle ear	,, spleen, glomeruli abnormal
10	1955	Milner	F	41	2	Mouth, nose	Kidneys, spleen, pancreas, heart, not lungs
11	1955	Walton and Leggat ..	F	42	10	Nasal septum ? lung	Kidneys and spleen, lungs equivocal
12	1956	Thomas	M	40	14	Nose and palate	Lungs, kidneys

* Yellow areas were noted on the capsule of the spleen, but no histological details of these are given.

of the erythrocytes is held by McLetchie and Colpitts (1949) to be the primary abnormality, but Hutchison (1954) regards the underlying cause of the haemorrhages as still unknown. Quite a different view is taken by Steiner (1954), who maintains that essential pulmonary haemosiderosis is an allergic disease, in which the pulmonary alveoli represent the shock tissue, and that the lung capillaries react by dilatation and increased permeability in an attack. The fact that the condition was relieved by splenectomy in his case is possibly a confirmation of his view, but the nature of the primary sensitizing agent was not discovered. It may be added here that in the present case the diagnosis of hypersplenism had been suggested and splenectomy was to have been undertaken but for the patient's terminal collapse.

The present case, although obviously not one of Ceelen's haemosiderosis, suggests a possible mechanism for that disease, and one which has apparently not as yet been suggested. On the whole it would seem that the source of the pulmonary haemorrhages in Ceelen's disease has not yet been established, and it is possible that the source tends to be hidden in the mass of blood, fibrin, siderophores, and reactive fibrosis. In the present case the lung in large part presented this same confusing picture, but in it were observed definite examples of arteritis. Indeed, even if these lesions had not been found, their existence could reasonably have been postulated on the basis of the similarity of this case with the reported cases of Wegener's granulomatosis. The hypothesis now offered that hyperergic arteritis may underly the condition of Ceelen's haemosiderosis receives some confirmation from the fact that in one of the reported cases of this disease pulmonary arteritis was actually observed; this was in a girl aged 7 (Anspach, 1939) with a three-year history of attacks of cough, haemoptysis, dyspnoea, pallor, and fever up to 100° F. Radiologically there was diffuse clouding of both hilar areas, and at necropsy the lungs showed haemorrhages, haemosiderosis, fibrosis, and necrotizing arteritis. The kidneys were not examined. In two of the cases of Wyllie *et al.* (1948) the kidneys were found to be normal, as they were also in Hutchison's case (1954). There is thus no real evidence to suggest that Ceelen's haemosiderosis is an incomplete form of Wegener's granulomatosis, but the present study of this latter condition does appear to offer incidentally a clue to the mechanism of Ceelen's haemosiderosis.

Summary

A case of Wegener's granulomatosis is described with clinical, pathological, and necropsy findings. The discussion is concerned with the recognition of this entity as a distinct variety of polyarteritis nodosa and its relationship to Ceelen's haemosiderosis.

I should like to thank Dr. Joseph Benn for permission to publish this case and Professor A. C. Lendrum for his kind and helpful criticism as well as for the photograph, Fig. 10.

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FACTORS AFFECTING THE RATE OF COUPLING OF BILIRUBIN AND CONJUGATED BILIRUBIN IN THE VAN DEN BERGH REACTION

BY

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Many workers have modified van den Bergh's method for determining bile pigment in serum (With, 1954). Two of the more satisfactory methods are those of Malloy and Evelyn (1937) and King and Coxon (1950). The present study was undertaken in order to explain differences in the results given by these two methods, and in an attempt to combine the desirable features of each, on a scale which was suitable for the day-to-day control of plasma bilirubin concentrations in newborn infants.

Methods

Serum.—Serum containing unconjugated, indirect-reacting bilirubin was obtained from infants at exchange transfusion. Serum containing conjugated bilirubin giving the direct reaction was obtained from patients with jaundice due to stones, cirrhosis, chlorpromazine, hepatitis, and also from infants with the "inspissated bile syndrome."

Diazo Reagent.—In most of the published procedures for determining bilirubin, the diazo reagent was prepared from 10 ml. of 0.1% (w/v) sulphanilic acid in 0.15 (or 0.25) *N*-HCl, to which 0.3 ml. of 0.5% (w/v) $NaNO_2$ was added. This mixture, containing 38% of the theoretical nitrite requirement of the sulphanilic acid, is referred to as the conventional diazo reagent. In preparing diazo reagents with other concentrations of diazotized sulphanilic acid, sulphanilic acid and at various reactions, the diazotization has always been conducted at a *p*H below 2.5.

Determination of Rate of Coupling of Bile Pigments.—The serum (0.1 ml.) was pipetted into 2.7 ml. water in the 1 cm. cell of the colorimeter. At zero time 0.7 ml. of freshly prepared diazo reagent (containing the required amount of 1.0% sulphanilic acid, 0.5% sodium nitrite, HCl, and water) was added and the contents mixed. The resultant colour was read in the colorimeter at 30 sec., 1 min., and at suitable short intervals up to 30 min. At 30 min., 3.5 ml. methanol was added, the solution mixed, and the colour read, as before, at 30 sec., 1 min., etc., up to 30 min. A blank was determined on 0.1 ml. serum diluted with 2.7 ml. water by adding 0.7 ml. of the diazo reagent without nitrite and measuring the colour before and after the addition of methanol. In the results given in the figures account has been taken of the effect of solvent and *p*H on extinction. The final standard was bilirubin.

Diazo Pigments.—To compare the absorption characteristics in different solvents at various reactions a standard of azo-bilirubin was prepared. Bilirubin (B.D.H.), 2 mg., was dissolved in 1 ml. of boiling $CHCl_3$, 8 ml. of ethanol was added, and 1.4 ml. of concentrated diazo reagent (1 ml. 1% sulphanilic acid, 0.1 ml. *N*-HCl, and 0.3 ml. 0.5% $NaNO_2$). After 45 min. the reaction was stopped with 100 mg. of ascorbic acid in 0.5 ml. of water. After centrifuging, the clear supernatant was stored at −12° C. Samples for study were taken to dryness *in vacuo* and dissolved in various solvents.

The diazo pigment of bilirubin diglucuronide (azobilirubin glucuronide) was prepared from 5 ml. of gall bladder bile by adding 88 ml. of water and 7.2 ml. of diazo reagent (6.0 ml. of 1% sulphanilic acid, 1 ml. of *N*-HCl, and 0.18 ml. 5% $NaNO_2$) followed after 10 min. by 50 g. of $(NH_4)_2SO_4$ and 2 ml. of *n*-butanol. The mixture was cooled to 4° C., spun at 1,000 *g*, and the pigment cake removed and dried *in vacuo* for 30 min. It was extracted with 10 ml., 2.5 ml., and 2.5 ml. portions of methanol which were combined, and evaporated *in vacuo*. Brown contaminants were separated by counter current distribution in a system of $CHCl_3$-methanol-0.2*M* acetate buffer at *p*H 4 (4:8:5, by vol.). To recover the pigment the system was saturated with $(NH_4)_2SO_4$, the aqueous layers were taken to dryness *in vacuo*, extracted with methanol, and the extract was dried. Samples were taken into the solvents to be examined.

Colorimetry.—Most of the studies of rates of colour development were made with a "unicam" S.P. 300 colorimeter using Ilford filter No. 404 ($\lambda_{max.}$ 525 mμ) and 1 cm. cells. The spectral curves of azo pigments were obtained with a "unicam" S.P. 500 spectrophotometer using 1 cm. cells. The *p*H of solvents is that recorded with a glass electrode. In calculating

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the results of experiments allowance has been made for colour differences due to *p*H and solvent.

Recommended Method for Infants.—The infant's heel is swabbed with 0.01% ethanolic acridine, smeared with vaseline, and punctured with a surgical needle. About 0.6 ml. blood is collected (5–10 min.) in a 6 × 50 mm. tube containing dry heparin (1 drop of 5,000 i.u. heparin delivered from a No. 12 needle). The tube, which is protected from strong light, is spun and the plasma separated.

The plasma (0.2 ml., or 0.1 ml. of plasma and 0.1 ml. of water, if a value over 15 mg. per 100 ml. of total pigment is expected) is pipetted into 5.4 ml. of water and mixed. Half of the material (2.8 ml.) is transferred to a second tube for a blank. Into the first tube 0.7 ml. of freshly prepared diazo reagent (10 ml. of 1.0% sulphanilic acid in 0.2 *N*-HCl, to which 0.3 ml. of 0.5% $NaNO_2$ has been added) is delivered, and into the second 0.7 ml. of the sulphanilic acid in HCl (without nitrite). The contents are mixed and read after 5 min. in a colorimeter (green filter, preferably Ilford No. 404 or 625), together with a standard. The solutions are returned to their tubes. The conjugated bilirubin is calculated from the formula $(E_{test} - E_{blank})/E_{standard} \times 10 \times 1.05$. To each tube is added 3.5 ml. of methanol. After mixing they are read in 5 min. The total pigment is calculated from $(E_{test} - E_{blank})/E_{standard} \times 20$.

A standard is prepared by weighing 10 mg. bilirubin into a hard glass volumetric flask, refluxing with chloroform until dissolved, cooling, and making to 100 ml. with chloroform. It is kept in the dark at 4° or −12° C. The standard solution (0.2 ml.) is pipetted into 3.5 ml. methanol, mixed with 0.7 ml. diazo reagent, and 2.6 ml. of water is added. The colour is read after 5 min. Water is used as a blank.

For a day-to-day check on the photometer variation a "standard" of methyl red (Haslewood and King, 1937) or Thompson's neutral grey solution (King and Wootton, 1956) may be used with the green filter. The factor which is suitable for the methyl red solution under King's conditions is not suitable under the more acid conditions described above. If this standard were used a new factor would have to be determined for each combination of instrument and filter.

Results

Factors Affecting the Light Absorption of Diazo Pigments.—Aqueous solutions of azopigment from bilirubin behaved according to Beer's law up to an extinction of 0.71, when an Ilford filter No. 404 was used. The wavelength of maximal absorption ($\lambda_{max.}$), and the relative extinction ($E\lambda_{max.}$) at this wavelength, of azobilirubin (Table I) and azobilirubin glucuronide, varied with *p*H and the solvent composition of the medium.

TABLE I

WAVELENGTH OF MAXIMUM ABSORPTION ($\lambda_{max.}$) AND RELATIVE EXTINCTION OF AZOBILIRUBIN IN VARIOUS SOLVENTS

Solvent	$\lambda_{max.}$ (mμ)		Relative Extinction at $\lambda_{max.}$		Relative Extinction Using Ilford 404 Filter	
	*p*H 1·7	*p*H 3·5	*p*H 1·7	*p*H 3·5	*p*H 1·7	*p*H 3·5
Water	560	520	100	65	100	73
50% methanol ..	559	522	106	100	105	92
68% ethanol ..	543	523	99	96	100	87
76% " ..	543	523	99	102	99	88
90% " ..	532*		—	90*		90*

* Determined at *p*H 2.

Protein affected the spectral characteristics. Bovine albumin (1%) shifted $\lambda_{max.}$ of azobilirubin at *p*H 1.5 from 560 mμ to 536 mμ, and reduced the $E\lambda_{max.}$ by 25%. With 0.2% albumin the change was almost as great, and 0.025% albumin reduced $E\lambda_{max.}$ by 13%. At a less acid reaction (*p*H 3.5) protein moved $\lambda_{max.}$ from 517 mμ to 524 mμ and $E\lambda_{max.}$ was increased by 10%. Human albumin and globulin had a similar effect to bovine albumin at *p*H 1.7 and *p*H 3.5. Bovine globulin, at *p*H 1.7, produced a slightly greater effect than albumin, while at *p*H 3.5 it had little influence. The effect of protein (1.0% albumin, *p*H 1.5) was abolished by 50% methanol. The spectral characteristics of azobilirubin glucuronide were less affected by protein than was azobilirubin.

Rate of Coupling of Bilirubin.—Sera of newborn infants, which have been shown by chromatography to contain mainly unconjugated bilirubin, were examined in the van den Bergh reaction before and after the addition of an equal volume of methanol. The rate of coupling was determined at *p*H 1.5, with varying amounts of the conventional diazo reagent (Fig. 1) and of undiazotized sulphanilic acid (Fig. 2). The rate of coupling at *p*H 3.5 is given in Fig. 3.

We examined the effect of using different quantities of sera in estimating the amount of pigment coupling in 30 min. in aqueous solution, and also on the total amount of pigment coupling in 50% aqueous methanol. With sera of newborn infants the calculated total pigment remained relatively constant as the serum volume was reduced, but the relative amount coupling in aqueous solution, i.e., the direct reacting component, appeared to increase, particularly at serum dilutions greater than 1 in 20. For example, one serum (total pigment 14 mg./100 ml.) appeared to have 0.7 mg. of direct reacting pigment (30 min.) when 1 ml. was used for a test in a final volume of 3.5 ml. A fivefold dilution doubled the amount

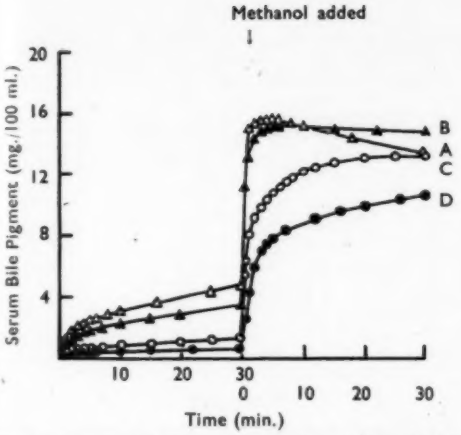

FIG. 1.—The effect of different concentrations of diazosulphanilic acid on the rate of coupling of bilirubin in serum at *p*H 1.5. The concentration of diazosulphanilic acid was: curve A, 0.052%; B, 0.023%; C, 0.0075%; D, 0.0037%. The concentration of undiazotized sulphanilic acid was twice that of diazosulphanilic acid.

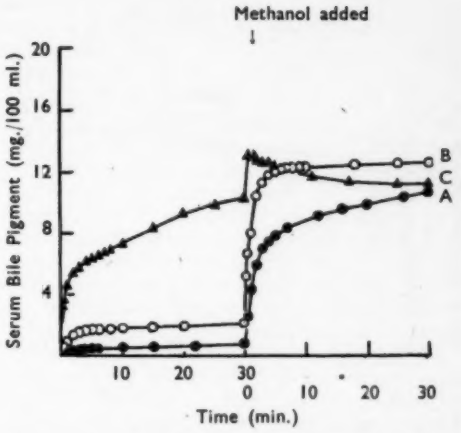

FIG. 3.—The rate of coupling of bilirubin in serum at *p*H 1.5 and 3.5, with low and high concentrations of diazotized sulphanilic acid. The excess sulphanilic acid was twice the concentration of the diazotized sulphanilic acid. The following were the *p*H values and the concentrations of diazotized sulphanilic acid before the addition of methanol: curve A, *p*H 1.5, 0.0037%; B, *p*H 3.5, 0.0037%; C, *p*H 3.5, 0.052%.

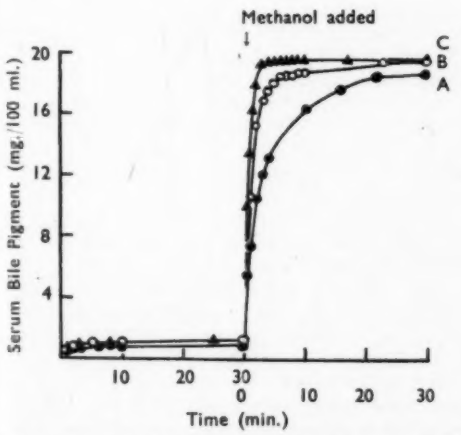

FIG. 2.—The effect of varying concentration of excess sulphanilic acid on the rate of coupling of bilirubin in serum at *p*H 1.5. The concentration of diazosulphanilic acid was 0.007% throughout. The concentration of the undiazotized sulphanilic acid, before the addition of methanol was: curve A, 0.013%; B, 0.053%; C, 0.193%.

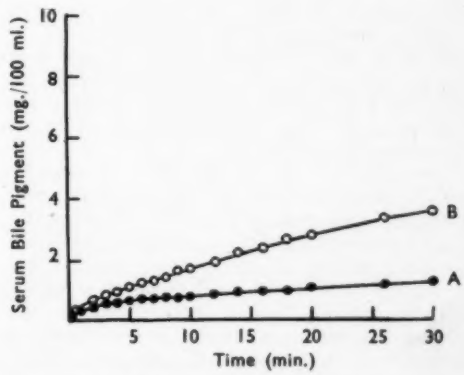

FIG. 4.—The rate of coupling of serum bilirubin in the presence and absence of added human serum protein. The initial reaction mixture (curve A) contained 0.1 ml. icteric serum, 0.007% diazotized sulphanilic acid, 0.013% excess sulphanilic acid, at *p*H 1.5. Curve B contained, in addition, 0.4 ml. of normal human serum. In calculating the amount of colour formed in curve B that due to the normal serum alone has been subtracted.

coupling in water and a twentyfold dilution increased it by 4.5 times. This effect was several times greater when the dilutions were made in normal serum, the protein being kept constant. This enhancement of the relative amount of coupling in aqueous solution could also be demonstrated by the addition of normal serum to a reaction mixture in which the amount of pigmented serum was constant (Fig. 4), provided that the protein content was initially low (not much higher than 0.2 ml. in 3.5 ml.). The effect could not be reproduced with human and bovine albumin, but it was given by human and bovine sera and by globulin.

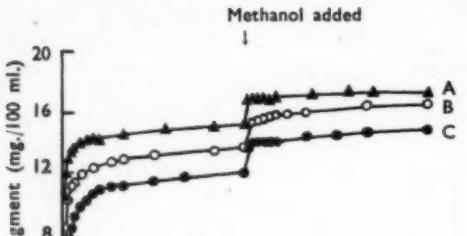

FIG. 5.—The rate of coupling of conjugated bilirubin in serum at *p*H 1.5, using different concentrations of diazosulphanilic acid. The excess sulphanilic acid was twice the concentration of diazosulphanilic acid. The following were the concentrations of diazotized sulphanilic acid before the addition of methanol: curve A, 0.023%; curve B, 0.0075%; curve C, 0.0037%.

Rate of Coupling of Conjugated Bilirubin.—Sera in which the main pigment was conjugated bilirubin were examined at *p*H 1.5 with varying amounts of the conventional diazo reagent (Fig. 5). An increase in the undiazotized sulphanilic acid from 0.008 to 0.046% did not alter the rate of coupling of conjugated bilirubin in water and 50% methanol significantly.

In a series of tests the apparent total pigment and the direct-reacting (30 min.) pigment were determined, using 1 to 0.05 ml. of pigmented serum which contained much conjugated bilirubin. The final volume of the aqueous mixture was 3.5 ml. The apparent total pigment remained relatively constant as the serum volume was reduced, and the apparent rise in direct-reacting pigment was attributable to the effect of dilution on the coupling of unconjugated bilirubin in aqueous solution. If the protein concentration was kept constant at 1 ml. by adding normal serum the 30 min. direct pigment increased slightly as with bilirubin, but there was a marked drop in the apparent total pigment (12.2, 10.1, and 8.8 mg. with 0.2, 0.1, and 0.05 ml. of pigmented serum). Protein had no effect on the rate of coupling of conjugated bilirubin in aqueous solution.

Results Compared by Recommended and King and Coxon's Methods.—Ten sera have been analysed by both methods, and the results are given in Table II.

TABLE II

TOTAL PIGMENT LEVELS COMPARED IN NEONATAL SERA DETERMINED BY KING AND COXON'S AND THE RECOMMENDED PROCEDURES

Serum No.	1 Bile Pigments Determined by King and Coxon Method (mg./100 ml.)	2 Bile Pigments Determined by Recommended Method (mg./100 ml.)	Comparative Values (1 as % of 2)
1	15·6	16·3	96
2	12·7	13·3	95
3	9·9	9·7	102
4	9·6	10·4	92
5	9·0	10·0	90
6	8·9	10·0	89
7	5·9	6·7	88
8	5·6	6·4	88
9	5·3	6·1	87
10	3·4	4·5	76

Discussion

Conditions for Distinguishing between Direct and Indirect Reactions.—Van den Bergh (1918) used the term "direct" in referring to a rapid colour development which occurred when diazotized sulphanilic acid was added to some sera in the absence of alcohol. "The direct reaction occurs in all cases in a few seconds, at the most 30 seconds." He distinguished this from the slow reaction in the absence of alcohol—". . . a reaction also occurred without addition of alcohol, but this commenced considerably later—there was an interval of 2, 3, 4 minutes or longer before the reaction began to become definite and still longer until it reached its greatest intensity." He identified this with the "indirect" reaction.

Nevertheless, others were less critically observant, and it was frequently held that bilirubin did not couple at all in simple aqueous solution, in this respect differing qualitatively from the direct-reacting pigments, which are now known to be conjugated bilirubin. A review of the conditions of the original van den Bergh reaction, and some of its modifications (Table III), indicates that there have been extensive changes in the conditions under which the reaction is carried out in aqueous solution, and some authors have not taken account of the way this affects the rate of coupling of bilirubin and of conjugated pigments.

Figs. 1–3 show that a sharp distinction between the rate of reaction of bilirubin and of conjugated bilirubin in aqueous solution can be drawn only at a low *p*H and a low concentration of diazotized sulphanilic acid. Bilirubin may react almost completely in 30 min. in aqueous solution at *p*H 3.5 and 0.052% diazosulphanilic acid (Fig. 3, curve C). These conditions must be avoided or a substantial error in estimating the proportion of unconjugated and conjugated bilirubin may result.

TABLE III

CONDITIONS UNDER WHICH BILIRUBIN AND CONJUGATED BILIRUBIN HAVE BEEN DETERMINED

	Reaction	Serum (%)	Diazotized Sulphanilic Acid (%)	Excess Sulphanilic Acid (%)	*p*H
van den Bergh and Snapper (1913)	Total	29	0·007	0·013	2·0
van den Bergh and Muller (1916)	Direct	31 or 44	0·004 or 0·003	0·007 or 0·005	5·3
Malloy and Evelyn (1937)	Direct	4	0·0037	0·0063	1·5
	Total	4	0·0037	0·0063	2·1
Ducci and Watson (1945)	Direct	4	0·0037	0·0063	1·5
	Total	4	0·0037	0·0063	2·1
Lawrence and Abbott (1956)	Total	1·66	0·003	0·0055	1·8
King and Coxon (1950)	Direct	10	0·0018	0·0032	2·6
	Total	10	0·0018	0·0032	4·2
White and Duncan (1952)	Total	4	0·011	0·019	1·8
Hsia *et al.* (1956)	Direct	1·66	0·006	0·044	1·5
	Total	0·83	0·003	0·022	1·8
Recommended method	Direct	2·86	0·007	0·193	1·5
	Total	1·43	0·0035	0·097	1·8

Figures in the body of the table are the final concentrations attained.

Aqueous Reaction of Bilirubin.—It is observed in Figs. 1, 2, and 3 that neonatal sera shown by chromatography to contain negligible conjugated bilirubin will couple with aqueous diazosulphanilic acid at *p*H 1.5 in two phases, a fast (0–3 min.) and a slow (3–30 min.). Both phases of the aqueous reaction of bilirubin can be increased by high diazosulphanilic acid concentration as in Fig. 1, curves A and B (under similar conditions to those of White and Duncan, 1952), and by a high *p*H (Fig. 3, curve C) until they amount at 30 min. to more than 70% of the total. In view of this fact, it seems probable that some or all of the small amount of colour which is developed at *p*H 1.5 and 0.0037% diazosulphanilic acid (conditions used by Malloy and Evelyn, 1937, as in Fig. 1, curve D) is also due to bilirubin and not to conjugated bilirubin.

Protein has its main effect on the slow phase of colour development (apparent after 3 min. in Fig. 4). A comparison of the aqueous reaction curve C of Fig. 1, which was obtained with a modification of the Malloy and Evelyn method previously used in this laboratory and circulated privately, and curve C of Fig. 2, obtained with the modification suggested in this paper, shows that in water high concentration of undiazotized sulphanilic acid affects neither the fast nor the slow phases of bilirubin coupling.

Although *p*H 1.5 is preferable to 3.5 for reducing the aqueous coupling of bilirubin (compare curves A and B of Fig. 3) even at the low *p*H about 4% of the bilirubin couples in 30 min. when the diazosulphanilic acid is 0.0037% (Fig. 1, curve D) as in the technique of Malloy and Evelyn (1937). These authors were largely responsible for the introduction of 30 min. as a period for the direct reaction, though there is nothing in the rate of colour development of conjugated bilirubin (Fig. 5, curve C) to make this period distinctive. A period of 30 min. was selected in order to include most of the direct pigment, though the authors mentioned that there was a slow development of colour over some hours. Ducci and Watson (1945) worked under similar conditions to Malloy and Evelyn and suggested the use of a "1 min. direct" to exclude the slow phase of bilirubin conjugation in water. It appears from curve C of Fig. 5, which was studied under the conditions of Ducci and Watson, that 1 min. is too short a time to measure the extent of the fast component due to conjugated bilirubin. It has a second disadvantage that the extinction is changing so rapidly that reproducible results would be difficult to obtain. It seems desirable that the time selected for the direct reading should include coupling of as little of the bilirubin and as much of the conjugated bilirubin as possible. We suggest that the colour development at 5 min. be taken as an estimate of the amount of conjugated bilirubin. In sera containing predominantly conjugated pigment the difference between the values at 5 and 10 min. is less than 10% of that at 10 min. (Fig. 5, curve B).

It might be thought that in the determination of direct-reacting pigment according to King and Coxon (1950) substantial amounts of bilirubin would react, as their usual procedure for total pigment is carried out at *p*H 4.2. This has been avoided, however, since the omission of $(NH_4)_2SO_4$ and ethanol reduces the *p*H to 2.6, where bilirubin is much less reactive.

Effect of Changes in Spectral Characteristics.—The differences in spectral curves and extinctions, produced by variations in solvent and *p*H (Table I), as well as protein concentration, indicate that caution is needed in using a standard under conditions which differ from the test for which it was designed. Thus the methyl red standard of Haslewood and King (1937) matches the colour of azobilirubin in their test (*p*H 4.2) but not that of the azobilirubin in 50% methanol under the more acid conditions (*p*H 2.1) of the Malloy and Evelyn test. Nor is the use of a filter satisfactory, since this will vary from instrument to instrument. If one departs from the conditions of King's test

it is essential to calibrate the method with bilirubin.

Effect of Protein. — Protein may alter the spectral characteristics of azobilirubin and the rate of reaction of bilirubin. In aqueous solution at *p*H 1.5, serum protein (1–3%) reduced the colour by 10%, or by 15% if 0.8 ml. of serum was used in the Malloy and Evelyn test. However, at *p*H 2.6 (direct reaction of King and Coxon, 1950) the extinction is scarcely altered by protein. The total amount of pigment shown by the Malloy and Evelyn test is approximately correct, because the effect of protein is abolished by the presence of 50% methanol.

Under special circumstances (Fig. 4) serum protein will facilitate the colour reaction of bilirubin in aqueous solution. It shares this property with many polar substances, e.g., ethanol, but the effect is minor, and cannot account for the "direct" reaction, as some authors have suggested. It is noted in Fig. 4 that the protein effect is negligible during the rapid phase of aqueous coupling of bilirubin (0–3 min.) and for this reason does not invalidate the suggested method which is based on a reading at 5 min.

The only circumstance in which dilution is likely to alter substantially the amount of apparent direct-reacting pigment would be the use of very small amounts (0.05 ml.) of serum in a modified Malloy and Evelyn technique. This would double the apparent conjugated pigment, and might erroneously suggest the development of obstruction in the infant.

Conjugated Bilirubin by King's Method

It may be anticipated that laboratories will select a method on the basis of its general usefulness, rather than its adaptability to the needs of the newborn infant alone. Since most determinations are made on adult sera, many of which have low concentrations of pigment, the King and Coxon method (Table III) has the great advantage of a low serum dilution. It has not been found possible to devise a method for determining conjugated bilirubin under King's conditions, because turbidity can only be overcome at a low *p*H, which makes the colour match with the methyl red standard unsatisfactory. If water is used in place of ammonium sulphate and ethanol, as suggested by King and Coxon (1950) for the determination of direct-reacting pigment, turbidity is frequently so great that a "blank" determination is insufficient to compensate for it. To avoid these difficulties the following technique may be used.

One millilitre of serum (or a mixture of serum and buffer) is mixed with 0.5 ml. of the conventional diazotized sulphanilic acid (*p*H now 2.2) and after 5 min. (longer will produce a false high direct reaction) 0.1 ml. of 5% ascorbic acid (or a knife tip, since an excess of up to 50 mg. may be used) is added to destroy the remaining diazotized sulphanilic acid. The procedure may then be continued in the usual way with $(NH_4)_2SO_4$ and ethanol, as bilirubin will not be coupled. This is recommended for laboratories which routinely use King's method, but occasionally need to know the actual amount of bilirubin and conjugated bilirubin in infant sera or in those of adults.

Recommended Rapid Procedure

For clinical use, and particularly for directing the treatment of jaundiced newborn infants, it is desirable to have a rapid method for estimating both bilirubin and conjugated bilirubin. The method should be accurate at 10 to 30 mg. of bilirubin/100 ml. of serum and should provide a compensation for the haemolysis which is frequently found in heel prick specimens of infant blood.

Although at *p*H 1.5 and 0.007% diazotized sulphanilic acid the aqueous reaction, which includes almost all of the conjugated pigment, could be read at 5 min., bilirubin coupling being minimal (Fig. 1, curve C), the coupling of unconjugated pigment in 50% methanol would be too slow. This could be accelerated by adding more diazotized sulphanilic acid, but would give a false high direct reading (Fig. 1, curve B) as in the White and Duncan (1952) procedure. This disadvantage can be overcome by increasing the excess sulphanilic acid to 0.19% rather than by increasing the diazotized sulphanilic acid. This accelerates the reaction of bilirubin in methanol but not in water (Fig. 2, curve C). The amount of apparent conjugated bilirubin was increased by less than 10%. For this reason it is recommended that the diazo reagent be prepared from 1% sulphanilic acid, but only the usual amount (0.3 ml./10 ml.) of nitrite should be added, as this gives a suitable concentration of both diazotized and undiazotized sulphanilic acid in the final mixture.

In jaundiced newborn infants the apparent conjugated bilirubin given by this method seldom exceeds 1.5 mg./100 ml. This is probably an artifact, due to coupling of bilirubin, and may be ignored. A final understanding of the significance of the minor proportion of indirect-reacting pigment, shown by the van den Bergh test in adult sera, must await a more detailed investigation of pigment I (bilirubin monoglucuronide) which is

usually the chief pigment of these sera. Although it gives a direct reaction, qualitatively, there is some evidence that an increase in colour development results when methanol is added (Billing, Cole, and Lathe, 1957). This may possibly account for some of the complicated time curves found in the literature.

The results given by this method are approximately 5–15% higher than those by the method of King and Coxon (1950) (Table II). This difference, which is present after account has been taken of *p*H and solvent effects, is due to incomplete coupling in King and Coxon's method. This effect of reagent concentration on the total amount of pigment which couples is shown in Fig. 1, curves B and C, for bilirubin, and in Fig. 5, for bilirubin glucuronide. The method which is recommended here also gives results for total pigment which are low, probably by about 3%. However, this cannot be corrected by increasing the concentration of diazo reagent, without producing greater errors in estimation of the conjugated bilirubin and producing fading in methanol (Fig. 1, curve A).

In the recommended method colour development (total pigment) may not be complete at 5 min. if the concentration of bilirubin exceeds 15 mg. per 100 ml. This can be overcome by starting with 0.1 ml. of serum, or reading the final colour until it is constant (7–9 min.).

Summary

The effect of *p*H, solvent, and protein on the spectral characteristics and optical extinctions of azobilirubin and azobilirubin glucuronide has been examined. The extinction was increased in aqueous solution by a drop in *p*H and was further raised by the addition of methanol.

The influence of *p*H, protein, concentration of diazotized sulphanilic acid, and of sulphanilic acid on the rate of coupling of bilirubin and conjugated bilirubin has been investigated. The rate of coupling of bilirubin in water and methanol was increased by a rise in diazo concentration and in *p*H.

A high concentration of undiazotized sulphanilic acid increased the coupling of bilirubin in 50% methanol at *p*H 1.8 without significantly increasing the rate of reaction in water.

A rapid method for estimating conjugated and unconjugated bilirubin in a small amount of serum has been described.

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AMINO-ACIDURIA ACCOMPANYING UNTREATED PERNICIOUS ANAEMIA AND SUBACUTE COMBINED DEGENERATION OF THE SPINAL CORD

BY

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Weaver and Neill (1954) reported abnormal excretions of taurine, sometimes accompanied by over-excretion of lysine, cystine, and leucine, in the urine of five patients suffering from pernicious anaemia. In one patient with subacute combined degeneration of the spinal cord in whom the blood picture was normal, they found that taurine alone was excreted in abnormal quantities. After treatment with vitamin B_{12} or liver extract, the urinary amino-acid patterns reverted towards normal. They employed the two-dimensional chromatographic method of Datta, Dent, and Harris (1950), using a quantity of urine containing 250 μg. of nitrogen applied to papers 15 cm. square.

Keeley and Politzer (1956) reported a similar type of amino-aciduria occurring in an African with untreated pernicious anaemia and showed that in 11 cases of megaloblastic anaemia associated with pregnancy and with malnutrition there was no evidence of an abnormal amino-aciduria.

These findings are of considerable interest since urinary chromatography could provide a useful means to differentiate between these anaemias and, as Weaver and Neill suggest, might help in the early diagnosis of subacute combined degeneration of the spinal cord, especially when haematological changes are minimal or absent.

The purpose of this paper is to report the results of the examination for amino-acids by means of chromatography of urine samples from nine cases of untreated pernicious anaemia, two having subacute combined degeneration of the spinal cord.

Clinical Features

The diagnosis of pernicious anaemia in Cases 1–9 (Table I) was based on the presence of macrocytic anaemia, megaloblastic bone marrow, histamine-fast achlorhydria, and the return of the peripheral blood and bone marrow to normal after adequate vitamin B_{12} therapy. There was no evidence of neurological involvements in seven of these patients. In Case 2 there was evidence of subacute combined degeneration of the cord, whilst Case 9 presented with this condition and showed only minimal haematological abnormalities. This case has been fully reported elsewhere (Robertson, Wood, and Joske, 1955). The age, sex, and haemoglobin values of each patient before treatment are given in Table I.

TABLE I

AGE AND SEX OF PATIENTS, INITIAL HAEMOGLOBIN VALUES, AND ABNORMAL FEATURES OF URINARY AMINO-ACID PATTERN

Case No.	Age (Years)	Sex	Initial Hb (g. %)	Abnormal Features of Amino-acid Pattern
1	64	M	12·6	Trace of lysine
2	69	F	8·0	Increased excretion of taurine
3	63	F	7·0	Increased excretions of taurine and BAIB*
4	72	M	6·8	Traces of lysine and aspartic acid
5	62	F	6·6	Traces of tyrosine, phenylalanine, and leucine
6	48	F	4·9	Increased excretions of taurine and BAIB
7	71	M	4·9	Increased excretions of taurine and BAIB plus traces of valine and aspartic acid
8	68	M	2·5	Excretion of taurine and BAIB greatly increased
9	70	M	12·4	No abnormal features

* BAIB = β-aminoisobutyric acid.

Urinary Chromatography

In order to eliminate any possible hour-to-hour variations in the urinary amino-acid pattern throughout the day, samples were taken for chromatography from 24-hour collections from eight of the patients and stored at 4° C. under toluene. In Case 8 because of the severe anaemia (Hb 2.5 g. % on admission) a 12-hour collection only was possible before treatment was begun. Chromatography was repeated in six cases when satisfactory remissions had been produced with vitamin B_{12} therapy. Volumes of desalted urine containing 250 μg. nitrogen (micro-Kjeldahl) were applied to Whatman No. 4 papers 60 cm. square

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(Dent, 1951). After running in phenol, followed by a collidine/lutidine mixture, the chromatograms were sprayed with a 0.1% solution of ninhydrin in butanol and finally examined after 16 hours at room temperature. Duplicate runs were made after oxidation with ammonium molybdate and hydrogen peroxide to detect the presence of cystine and methionine. Unidimensional runs in collidine were examined for the presence of tyrosine and histidine after spraying with Pauly's reagent.

Results

The amino-acid pattern of the urine from the patient with subacute combined degeneration of the spinal cord and minimal haematological findings (Case 9) was normal and identical before and after treatment. It consisted of glycine as the predominating amino-acid with smaller excretions of taurine, serine, alanine, glutamine, cystine, β-aminoisobutyric acid (BAIB), and a trace of glutamic acid. Histidine was also present before and after treatment. These amino-acids were also detected in the urinary chromatograms from Cases 1–8 before and Cases 1, 4, 6, 7, and 8 after treatment, mainly in normal concentrations as assessed by the size and intensity of the resultant ninhydrin spots. The abnormal features of each urinary amino-acid pattern before treatment are set out in Table I. (Specimens of urine from Cases 2, 3, and 5 could not be obtained after treatment.)

Slightly increased outputs of several amino-acids appearing as traces on the chromatograms from Cases 1, 4, and 7 were no longer detectable after treatment. Excretions of taurine and BAIB, large initially in Cases 6, 7, and 8, returned to normal after treatment, and in each of the resultant chromatograms glycine became the predominating amino-acid.

Discussion

No characteristic urinary amino-acid patterns, common to each case of untreated pernicious anaemia with or without evidence of subacute combined degeneration of the spinal cord, were observed in this series. Of the patients presenting with haemoglobin values of 8 g.% or less, two showed minimal and dissimilar departures from the normal pattern (Cases 4 and 5), four increased outputs of taurine and BAIB (Cases 3, 6, 7, and 8), while Case 2 showed excessive taurine excretion alone. With such a lack of uniformity of pattern accompanying this degree of anaemia it seems very unlikely that urinary chromatography will aid in the diagnosis of those cases of pernicious anaemia presenting with minimal haematological abnormalities or equivocal neurological signs. The slightly increased excretions of lysine, aspartic acid, tyrosine, phenylalanine, leucine, and valine which resulted in small or faint spots on the chromatograms in Cases 1, 4, 5, and 7 initially and do not appear in the urinary patterns from normal persons when this technique is used (Dent, 1954; Walshe, 1953) could be attributed to mild hepatic dysfunction resulting from fatty infiltration which may accompany pernicious anaemia. An increased excretion of taurine in five out of seven patients with initial haemoglobins of 8 g.% or less confirms the observations of Weaver and Neill (1954), but at the same time it was not possible to detect excessive outputs of leucine, lysine, or cystine. Four of these patients in addition excreted large quantities of BAIB. Taurine or BAIB may be the predominating amino-acid excreted by normal persons (Dent, 1954; Walshe, 1953) and these two substances may occur together in abnormal quantities in the urine of patients suffering from acute or chronic liver disease (Walshe, 1953). It is unlikely that the marked increased excretion of these two substances in our cases was due primarily to liver damage, since excessive quantities of other amino-acids, especially cystine, were absent. Since vitamin B_{12} is concerned in methyl group synthesis and in transmethylation of homocysteine (Jukes, Stokstad, and Broquist, 1950), a lowered rate of synthesis of methionine due to vitamin B_{12} deficiency could result in an enhanced urinary excretion of taurine by degradation of accumulated homocysteine within the body, and it is of significance that in three patients after treatment with vitamin B_{12} the urinary excretion of this substance became normal. A similar situation in respect of thymine and nucleic acid synthesis (Glazer, Mueller, Jarrold, Sakurai, Will, and Vilter, 1954) could account for the enhanced excretion of BAIB, since likewise the urinary excretion of this substance returned to normal after treatment in the cases studied, but there is an alternative explanation. Sandler and Pare (1954) observed that urinary excretion of BAIB was increased early in experimental starvation of young volunteers, and the anorexia and general malaise accompanying severe pernicious anaemia predispose to a similar situation.

It is difficult to assess with confidence any but gross differences in concentrations of urinary amino-acids by means of two-dimensional paper chromatography, and, apart from laborious procedures involving ion exchange, there exists at present no reliable means of estimating BAIB in the urine. Recently Ling (1957) has introduced a method for estimating urinary taurine and it would

thus be possible to gain more precise data on the excretion of this substance in pernicious anaemia before and after treatment.

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A SCREENING TEST FOR STEATORRHOEA USING ^{131}I-LABELLED TRIOLEIN

BY

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During recent years there has been increasing interest in the study of fat absorption, particularly in the investigation of conditions associated with steatorrhoea. The need for a single diagnostic test for steatorrhoea, suitable for use with both out-patients and in-patients, has become apparent. Ideally, such a test should be easy to carry out for the patient and nursing staff as well as for the laboratory technician, and should distinguish, without equivocation, between steatorrhoea and other conditions. So far, no such test has been published, but the procedure described in this paper goes a long way towards meeting these requirements, being simple, easy, and convenient to carry out, and, on the whole, giving a definite answer about the presence or absence of steatorrhoea. In addition, there is given a convenient method for the preparation of neutral fat or fatty acid labelled with ^{131}I.

Principle of the Test

The patient, on a normal diet, is given a tracer dose of radioactive fat in milk, and faeces are collected for three or four days. The percentage of the administered dose excreted in the faeces is determined. Values over 6% are consistent with steatorrhoea, but it is better to regard 5% as the upper limit of normality and to carry out a chemical fat determination on the faeces when values between 5% and 7% occur. This can be done on the faeces used for the determination of radioactivity, thus wasting no extra time and calling for no extra effort on the part of the patient or nursing staff. It should be emphasized that this test is presented as a screening test for steatorrhoea, to replace conventional chemical procedures in most cases. It will not, in itself, distinguish the various causes of steatorrhoea from one another.

Technique of the Test

Preparation of ^{131}I-labelled Fat.—The reagents required are the following:

1. Potassium iodide solution, 1 mg./ml.
2. Potassium iodate solution, 2 mg./ml.
3. 2N sulphuric acid.
4. Iodide-thiosulphate solution. Dissolve 5 g. potassium iodide and 5 g. sodium thiosulphate in distilled water to give a volume of 100 ml.
5. Ether (anaesthetic grade).

All other reagents should be analytical reagent grade.

Preparation of Iodinating Solution.—To 1–2 ml. of a solution containing 1 mc. of carrier-free ^{131}I as sodium iodide,* in a 10 ml. glass-stoppered test-tube, add 1 ml. of potassium iodide solution, layer 3 ml. of ether on to the aqueous phase and then add 0.5 ml. of potassium iodate solution and 0.2 ml. of sulphuric acid. Stopper the tube and shake gently until all the liberated iodine has passed into the ether layer. Remove the lower, colourless layer with a Pasteur pipette and discard. Add drop-wise to the remaining ether layer a freshly prepared solution of chlorine in ether (made by treating potassium permanganate with concentrated hydrochloric acid and passing the resulting chlorine into ether until the ether is distinctly yellow) until the brown colour of the iodine has disappeared. As a rule, only one drop of chlorine solution is needed.

Iodination of Fat.—Dissolve 1 ml. of triolein in 3 ml. of ether in a 15-ml. glass-stoppered tube. Using a clean Pasteur pipette, add the iodinating solution prepared above, stopper, mix by tapping, and allow to stand at room temperature for at least one hour. The preparation may be left overnight at this stage, without harm. Then add an equal volume of iodide-thiosulphate solution, shake gently, allow the mixture to separate into two layers (centrifuge, if necessary, to break any emulsion) and remove the lower layer with a Pasteur pipette. Repeat the washing process with iodide-thiosulphate and then, twice more, with equal volumes of distilled water. Transfer the washed ethereal solution of iodinated fat to a 150 ml. glass-stoppered Erlenmeyer flask, evaporate the ether on a water-bath at about 60° C., add about 0.5 ml. of "Tween 80" (Solmedia Ltd.†) and 100 ml. of distilled

* Supplied by the Radiochemical Centre, Amersham.
† Solmedia Ltd., 35, Orford Road, London, E.17.

water, stopper and shake well until an even suspension is produced.

Assay of Radioactivity of Fat.—This may be carried out by any convenient method. In this work the multitube γ-ray counting apparatus of Veall and Baptista (1954) has been used, the fat suspension being diluted 1 in 10. The excreta counter described below has also been used, after calibration with 1 μc. of ^{131}I.

Preparation of Patient before Test.—In order to make possible the determination of the faecal fat by chemical methods, if this should prove necessary, on the specimens used for measuring radioactivity, the patient should be instructed to consume, for two days before the test starts and during the period of faecal collection, 2 pints of milk and 1 oz. of butter or margarine in addition to the other constituents of his usual diet. This will provide an intake of about 100 g. of fat daily. In addition, thyroid absorption of iodine should be blocked by the administration of 10 minims of Lugol's iodine t.d.s., or 10 mg. of potassium iodide (as tablets) t.d.s., for the two days preceding the test and the day of the test itself.

Administration of Dose.—The dose is most conveniently given about two hours after the patient has had his usual breakfast. An accurately measured volume (usually 2 ml., containing 5–10 μc. ^{131}I) of the freshly shaken fat suspension is delivered into about 100 ml. of milk in a cup. After the dose has been completely drunk, the patient drinks two further 100 ml. portions of milk, which are added to the cup. There is no significant radioactivity remaining in the cup, if this procedure is followed. For young children, the dose should be 1 μc. for each stone of body weight. The stock radioactive fat suspension may be kept at least two weeks at room temperature without deterioration or liberation of free iodine.

Collection of Faeces.—Patients should be instructed to collect all faeces, without waste and without contamination by urine, passed from the moment of drinking the dose until the end of four clear days, including the fourth day up to the time of administration of the dose. The collection period may be reduced by one day, with a risk of introducing small errors (Tables II and III).

Faeces are best collected in non-disposable spun-aluminium cans, with tight-fitting lids,* made to fit snugly into the excreta counter and to hold about 2 litres. They may be easily cleaned with detergent and hot water. The patient should be instructed to pass his faeces directly into the can, after first emptying his bladder. Both male and female patients have, as a rule, found no difficulty in using the cans in this way. Several days' collections may be made in the same can, but, if the patient objects to this, one container may be used for each day, in which case the cans must be labelled with the date of collection. The main advantages of this method of collection are that it is almost impossible to contaminate the faeces with urine (which would completely invalidate the results) and that there is no waste of faeces. If it is impossible to collect the faeces in this way, then more conventional methods may be used, care being taken to transfer the faeces quantitatively and to avoid urine contamination. Waxed cartons, although suitable for well-formed stools, have in practice not proved suitable for liquid or semi-formed stools and have, in addition, insufficient capacity for the bulky stools, often in excess of 1 kg., passed by patients with steatorrhoea or diarrhoea.

* Made by W. Still and Co., 29–31, Greville Street, London, E.C.1.

Measurement of Radioactivity.—This may be conveniently carried out using the excreta counter of Veall and Vetter (1952), which consists of a ring of Geiger tubes. The can should be raised so as to bring it into the sensitive zone of the tubes. At least 3,000 counts above background should be recorded, using conventional electronic apparatus or a clinical monitor.* This equipment is the same as that used in many laboratories for the urinary excretion test of thyroid function of Fraser, Hobson, Arnott, and Emery (1953). The activity of a standard must be determined at the same time as that of the faeces. The standard (usually 20% of the dose) is accurately pipetted into a can containing about 1 litre of warm 10% gelatin. This is allowed to set, the fat being stirred in with a glass rod left *in situ*. This procedure gives an even dispersal of the radioactive fat and prevents spilling of the standard and accidental contamination of the counter. Activity of the faeces is expressed as a percentage of the dose given.

Chemical Fat Determination.—Where necessary, this may be quickly carried out by the method of Kamer, Huinink, and Weyers (1949) on the same faecal specimens.

* Type 1294 DY, Dynatron Radio Ltd., Maidenhead, Berks.

Results

The test was carried out on six healthy males (laboratory staff) and 20 patients suffering from anaemia or disorders of the gastro-intestinal tract or pancreas, collection of faeces being made for three to six days. In order to determine the validity of the test, a chemical fat determination was made on the same sample of faeces used for measuring radioactivity in all patients, who consumed about 100 g. fat daily. No chemical fat determination was carried out in the case of the healthy normals, who were on their customary diet, absence of steatorrhoea being presumed.

In the six healthy males, excretion of radioiodinated fat did not exceed 4% of the dose administered (Table I) in three days. The results obtained with the patients fell into two groups, on the basis of the average daily excretion of fat, the dividing line being at 6 g. fat, corresponding to approximately 94% absorption of fat.

TABLE I
EXCRETION OF ^{131}I IN FAECES OF NORMAL SUBJECTS

% Dose Faecal ^{131}I Excreted in 3 Days*
4·0
3·5
3·4
3·0
3·1
1·7

* No simultaneous fat determination carried out.

In the group excreting below 6 g. fat daily (Table II), all 11 patients showed an iodine excretion not greater than 6%, in eight the excretion being below 5%, and in seven lower than 4%. On the basis of the fat excretion, all these patients must be considered as not showing steatorrhoea at the time of carrying out the test. Of the three patients with faecal radio-iodine excretions of more than 5%, two were elderly women who were unable to use the cans directly for collecting faeces and it is probable that some urinary contamination occurred. The third patient had severe ulcerative colitis. It is possible that, in this disease, there may be some leakage of iodide from the blood into the bowel lumen.

TABLE II
EXCRETION OF ^{131}I IN FAECES OF PATIENTS WITHOUT STEATORRHOEA

Disease	Days of Collection	Faecal Fat (g./day)	Faecal ^{131}I Excretion (% Dose)	
			Total	After 3rd Day
Hypochromic anaemia	4	5·3	3·9	<0·2
Diarrhoea	6	3·5	4·2	0·4
Hypochromic anaemia	3	3·5	0·6	—
Diarrhoea	4	2·9	3·7	0·4
Ulcerative colitis	4	2·7	5·6	0·2
Diverticulosis of small intestine	3	2·5	6·0	—
Hypochromic anaemia	4	2·5	0·5	<0·2
Megaloblastic anaemia	4	2·2	5·1	<0·2
Post-gastrectomy *	6	2·2	1·2	1·2
Diarrhoea	4	0·7	0·6	<0·2
Post-gastrectomy + megaloblastic anaemia	3	0·6	0·2	—

* No stools passed in first three days.

In the group excreting above 6 g. fat daily (Table III), radio-iodine excretion exceeded 6% in eight of the nine patients and exceeded 7% in six cases. In one instance, the radio-iodine excretion was just under 5%. In this patient, who had a gluten-sensitive steatorrhoea, the excretion of faecal radio-iodine was 1.6% of the dose in the first two days, 3.3% on the third day, and none on the remaining two days. This pattern of excretion is anomalous and is consistent with some of the stool not having been collected on the important first two days.

From these results it may be concluded that radio-iodine excretions below 5% exclude steatorrhoea and above 7% are consistent with steatorrhoea, in those cases where the test has been correctly carried out. With excretions between these values and whenever there is doubt about urinary contamination or completeness of collection of the faeces, a chemical determination on the same specimens of faeces will be necessary to decide the point.

TABLE III
EXCRETION OF ^{131}I IN FAECES OF PATIENTS WITH STEATORRHOEA

Disease	Days of Collection	Faecal Fat (g./day)	Faecal ^{131}I Excretion (% Dose)	
			Total	After 3rd Day
Chronic pancreatitis	5	50·7	29·5	0·4
Post-gastrectomy	4	48·4	80·3	<0·2
Resection of most of small intestine	4	30·8	22·7	0·2
Post-gastrectomy	6	18·7	7·2	1·0
"	5	12·2	6·9	0·2
Tropical sprue	6	11·2	6·1	<0·2
Idiopathic steatorrhoea	4	9·5	13·5	0·3
"	3	7·6	13·9	—
Gluten-sensitive steatorrhoea	5	6·8	4·9	<0·2

Discussion

The use of ^{131}I-labelled triolein in the study of fat absorption is not new. Stanley and Thannhauser (1949) administered olive oil containing 100 μc. ^{131}I and studied blood levels of radioactivity. They did not report any observations on the radioactivity of the faeces. Since then, most workers have endeavoured to find some relationship between blood radioactivity and disease, following doses of iodinated fat ranging from 25 to 100 μc. (Baylin, Sanders, Isley, Shingleton, Hymans, Johnston, and Ruffin, 1955; Malm, Reemtsma, and Barker, 1956; Sanders, Isley, Sharpe, Baylin, Shingleton, Hymans, Ruffin, and Reeves, 1956; Ruffin, Shingleton, Baylin, Hymans, Isley, Sanders, and Sohmer, 1956; Shingleton, Baylin, Isley, Sanders, and Ruffin, 1957; McKenna, Bourne, and Matzko, 1957; Beres, Wenger, and Kirsner, 1957). In a few cases, radioactivity measurements were made on the faeces and in still fewer cases parallel determinations of radioactivity and fat were carried out. Baylin and others (1955) reported a faecal excretion of less than 2% of the dose during a 48-hour collection in six normal subjects after 50 μc. of ^{131}I-labelled triolein. Sanders and others (1956) and Shingleton and others (1957) found less than 1.4% of the dose in 24 normals in a 48-hour collection after 25–50 μc. of radioactive fat, with greatly increased values in diseases clinically associated with steatorrhoea (chemical

fat determinations were not carried out). Ruffin and others (1956) found less than 2% of the dose in a 48-hour collection of faeces after 25 μc. of labelled fat in normals. McKenna and others (1957) reported a faecal excretion of less than 1.5% of the dose (50–100 μc.) in five normals in 48 hours, but found values up to 4% in seven normals in 72 hours, and increased values in diseases with steatorrhoea (confirmed chemically, however, in only two cases). Beres and others (1957) measured a three-day faecal collection, both for radioactivity and fat, after 40–100 μc. ^{131}I-labelled triolein. In eight subjects with fat absorption coefficients above 94%, faecal radio-iodine excretion was less than 1.6%, while in five patients with fat absorption of 90% or less, excretion ranged from 3.5 to 53.5% of the dose.

The level of excretion of labelled fat is lower in subjects without steatorrhoea in most of the published data than is reported here. This difference is probably related to the longer collecting period employed in our test. In normal subjects, who usually have one bowel action daily, a 48-hour collection period may not include the second stool if there is bowel irregularity during the course of the test, a not infrequent event. Further, it is well known to those who carry out daily faecal fat estimations on patients with and without steatorrhoea that there is a wide variation in the amount of fat excreted daily. To get an accurate picture of fat excretion, it is necessary to take the mean of several days' excretion. Examination of the results of daily faecal radioactivity measurements in our subjects shows that, while in many cases excretion is essentially complete in 48 hours, there are several cases where only about two-thirds of the excretion has taken place in this time. Usually, excretion is complete in four days, but a three-day collecting period may be used with only a little error. However, when the patient is constipated, a six-day collection period may be necessary. As it may be unreliable to carry out a chemical fat determination on a 48-hour collection of faeces, it is recommended that the collection period in this test should be a minimum of three days and preferably four days.

It will be noticed that, while chemical fat and radio-iodine excretion are correlated, the values for each may be markedly different in the same patient. It is not possible to convert radio-iodine excretion into faecal fat excretion by means of a factor, with any accuracy. This is because the two tests do not quite measure the same thing. The labelled fat test measures the ability of the gut to absorb a particular triglyceride and its products of digestion, while the chemical fat test measures the ability of the gut to absorb a mixture of fats (and derivatives) of varying molecular weight and degree of saturation. It is by no means certain that all fats, both saturated and unsaturated, can be absorbed equally well, particularly in disease. There is, therefore, no reason to expect that percentage fat excretion and percentage radio-iodine excretion should be identical. However, the experimental results reported here and by other workers show that there is a correlation between steatorrhoea and radio-iodine excretion.

It is theoretically possible that some of the radioactive iodine in the faeces may be derived from inorganic iodide resulting from metabolism of the labelled fat after absorption and subsequently excreted into the intestine; or iodine might be liberated from labelled fat in the intestine by enzymes or bacteria and thus be treated differently from the fat. However, Ruffin *et al.* (1956) have shown that the radioactive material in the stool is non-dialysable and that all the radioactivity is found in the fat fraction obtained by ether extraction. Further, Hoffman (1953) has shown, in animal experiments, that the isotope-fat bond is stable to enzyme action during absorption from the gut. It is unlikely, therefore, except perhaps in cases of severe diarrhoea or ulcerative colitis, that diffusion of radioactive iodine from the blood into the gut will be of any significance.

No attempt has been made in this paper to correlate levels of radio-iodine excretion with the cause of the steatorrhoea, as the amount of fat in the faeces is not diagnostic of any particular disease. The test is presented as a screening test, to be carried out instead of a fat balance, to select for further investigation those patients suffering from steatorrhoea, and those whose excretion lies in the border zone of 5 to 7%, thus necessitating a chemical determination. The test should prove of particular value in excluding steatorrhoea, especially in anaemia associated with absorption defects.

The test can also be carried out on infants and children, provided it is possible to keep the faeces free from urinary contamination. Napkins can be rolled up in a waterproof bag and the activity of the faeces in them read in the excreta counter. Because of the small dose given to infants, a long counting time is necessary.

The advantages of the test described in this paper are that it is easy to carry out and can be used for out-patients; an accurately known diet is not essential; the dose is given in a palatable

form; the laboratory techniques involved are quick, easy, and accurate; no blood samples are required. Tests of fat absorption depending on blood radioactivity often require three or more blood samples, taken several hours after the administration of the dose, which has to be large. This makes these tests uncomfortable and tedious for the patient, besides adding to the labour of the technician. Further, blood tests are almost impossible for very young children. By relying only on faecal radioactivity measurements, it has been possible to use a very small dose of ^{131}I, thus reducing considerably the radiation hazard, an important consideration when dealing with children.

The disadvantages of our test are that faeces have to be collected accurately; care has to be taken to prevent contamination by urine; on a few occasions a chemical fat determination might prove necessary. However, this causes extra work only for the technician. Perhaps the most serious disadvantage is that it requires the use of radioactive material and equipment to measure radioactivity. Those laboratories equipped to carry out urinary radio-iodine excretion tests, however, have all the apparatus necessary for the faecal labelled fat test.

Summary

1. A convenient method for labelling triolein (or oleic acid) with ^{131}I is described, and, also, the preparation of an emulsion suitable for oral administration.

2. A technique is given for administering 5–10 μc. of this fat and assaying the activity in stools collected for three or four days.

3. The results of carrying out this test on six normals and 20 patients, nine with steatorrhoea proved chemically, show that steatorrhoea may be excluded when there is an excretion of radio-iodine less than 5% of the dose. Values over 7% are consistent with the presence of steatorrhoea. For intermediate values, faecal fat may be determined by chemical methods, on the same faecal specimens, without the need to carry out another test.

4. The test will not diagnose the cause of steatorrhoea, but is presented as a screening test for steatorrhoea, to be carried out in place of the conventional fat balance.

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A COMPARISON OF DIRECT AND EXTRACTION METHODS FOR THE DETERMINATION OF T-1824 (EVANS BLUE) IN PLASMA AND SERUM

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The dye T–1824 (Evans Blue) has gained wide acceptance for determining plasma volume. Recently there has been a renewed interest in dye circulation studies for the measurement of cardiac output, central volume, cardiac shunts, and valvular regurgitation. The method of estimation most commonly used is spectrophotometric analysis of the dye-tinged plasma (or serum) compared with standard dye solutions prepared from the subject's plasma using a plasma blank (Chinard, 1951). Haemolysis and lipaemia introduce serious errors in these determinations (Gregersen, 1938). To overcome these factors, several extraction methods have been described (e.g., Allen, 1951 ; Bedwell, Patterson, and Swale, 1955).

To our knowledge no studies have been made on the relative values in routine clinical investigation of extraction and direct measurement of the dye in plasma (or serum). The purpose of this paper is to compare an acetone-extraction method with direct estimation in plasma and serum and to discuss their relative merits in clinical use.

Methods

A preliminary sample of venous blood was drawn from an indwelling needle to provide blank and standard solutions ; further samples were taken eight and 12 minutes after each injection of T–1824. The patients received two to four dye injections 15 to 30 minutes apart. Samples were placed in two dry, freshly siliconed tubes, one containing a small amount of powdered heparin as anticoagulant. Care was taken not to introduce bubbles or to cause excessive agitation when mixing. The mean concentration of the eight-minute and 12-minute samples was used in comparing the three methods described below.

A. Extraction Method.—A modification of the cellulose-absorption, acetone-elution method of Allen (1951) was used. One millilitre of a 50% aqueous solution of "teepol" 530‡ was added as a detergent to 2 ml. plasma, and the mixture was allowed to stand 10 minutes in a water-bath at 50° C. A 1 cm. cellulose§ column was prepared in a Gooch crucible ; the plasma was passed through this by gravity, and the column was then rinsed with teepol-saline, 1:200 (v/v) solution. After suction to remove the excess saline solution, the crucible was placed in a clean funnel over a 10-ml. volumetric flask and the dye eluted with 50% acetone-water heated to 50° C. in the water-bath. To start with, the acetone solution was added slowly but the rate was increased after dye appeared in the volumetric flask. After bringing to volume the optical density was determined on a "unicam" spectrophotometer at a wavelength of 620 λ using an acetone-water blank. As many as 10 samples can be analysed simultaneously in one to two hours.

The optical densities (O.D.) of two sets of duplicate standards were determined with each group of samples: standard No. 1, a known concentration of dye in the patient's plasma (usually 10 mg./l.), extracted with the other samples, and standard No. 2, an equal amount of dye (same pipette) brought to volume with acetone-water.

$$(1)\quad \frac{\text{O.D. extracted standard (1)}}{\text{O.D. non-extracted standard (2)}} \times 100 = \%\ \text{extraction}$$

The dye-plasma samples are corrected for the percentage loss during the extraction and expressed in mg./l.

$$(2)\quad \frac{\text{O.D. unknown}}{\text{O.D. standard No. 2}} \times \frac{100}{\%\ \text{extraction}} = \frac{\text{unknown (mg./l.)}}{\text{standard (mg./l.)}}$$

or simply:

$$(3)\quad \frac{\text{O.D. unknown}}{\text{O.D. standard No. 1}} = \frac{\text{unknown (mg./l.)}}{\text{standard (mg./l.)}}$$

Ordinarily, only standard No. 1 (known concentration of dye in patient's plasma for extractions), and equation (3) are used.

B. Direct Determination of Dye in Plasma.—After centrifugation, the optical density of the plasma was measured with the patient's plasma as a blank and

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† Member, external staff, Medical Research Council.

‡ Courtesy of Shell Petroleum Company.

§ Shredded "kleenex" in 0.9 N NaCl.

measured amounts of dye in the subject's plasma as standards. Cuvettes contained 0.7 ml.

C. **Direct Determination of Dye in Serum.**—The blood was allowed to clot and retract. If the clot adhered to the side of the tube it was gently freed with a thin glass rod. After centrifugation the specimens and standards were prepared and measured as in B.

Results

Before comparing the extraction method with the non-extraction techniques, it was necessary to determine the validity of the former. In addition to extracting 24 plasma samples with known amounts of T–1824 during the routine dye analysis of the patients studied, 77 plasma solutions of known and varied dye concentrations were analysed and the percentage extractions calculated.

The mean extraction of the 101 known samples, concentrations ranging from 3.85 to 20.0 mg./l., was 93.7% (standard deviation 2.6%). There was only a slight variation in extraction over the range of dye concentrations encountered in our studies that was not correlated with concentration (Table I). The percentage extraction varied from day to

TABLE I

PERCENTAGE EXTRACTION* FROM 5 TO 20 MG./L. DETERMINED ON FOUR SEPARATE DAYS

Date	O.D. Blank	Percentage Extraction 5 mg./l.	Percentage Extraction 10 mg./l.	Percentage Extraction 20 mg./l.	Percentage Variation
1	0	94·7	94·4	93·8	0·9
2	0	90·5	92·2	90·9	1·7
3	0	95·1	94·0	93·1	2·0
4	0	95·9	93·9	95·1	2·0

* All extractions and standards determined in duplicate.

day, mainly due to temperature changes, a higher extraction being found at higher temperatures; when allowance was made for this by analysis of variance, the standard deviation was reduced to 2.2%. For this reason, correcting a group of samples for the daily extraction percentage gave an accuracy within the narrower range.

In preliminary experiments markedly haemolysed and lipaemic plasma blanks were analysed and an optical density of zero was invariably recorded as evidence of the complete removal of pigments and turbidity during the rinsing process; also, after adding an equal amount of dye to haemolysed, lipaemic, and clear samples before extraction, the final optical density in all specimens agreed to within 2%.

As the extraction was reliable over the dye-concentration encountered in these clinical studies and was not influenced by haemolysis or lipaemia, it was used as a basis for comparing the direct

TABLE II

CONCENTRATION OF T–1824 OBTAINED BY DIRECT DETERMINATION IN PLASMA AND SERUM COMPARED WITH EXTRACTION VALUES CORRECTED FOR PERCENTAGE LOSS

Subject	Injection No.	Extraction (mg./l.)	Serum (mg./l.)	Percentage Difference	Plasma (mg./l.)	Percentage Difference
D.T.	1	6·13	—	—	6·01	− 1·9
	2	11·08	—	—	12·25*	+10·6*
A.F.	1	5·53	6·28*	+13·6*	5·53	0
	2	10·27	10·63	+ 3·5	10·60	+ 3·2
	3	15·32	15·53	+ 1·4	16·17	+ 5·5
W.S.	1	4·25	4·80*	−12·9*	4·59	+ 8·0
	2	8·71	9·47	+ 8·7	9·16	+ 5·2
	3	12·86	13·53	+ 5·2	13·68	+ 6·4
M.S.	1	4·77	4·40	− 7·8	3·95	−17·2
	2	9·60	9·10	− 5·2	8·89	− 7·4
	3	14·12	13·57	− 3·9	13·28	− 5·9
M.L.	1	4·54	4·27	− 5·9	4·87*	+ 7·3*
	2	11·35	11·47	+ 1·1	12·06	+ 6·3
A.P.	1	4·11	3·73	− 9·2	3·89	− 5·4
	2	8·15	7·77	− 4·7	7·57	− 7·1
	3	11·79	11·06	− 6·2	12·07	+ 2·4
P.B.	1	4·57	3·00†	−34·4†	4·16	− 9·0
	2	8·70	7·17†	−17·6†	8·11	− 6·8
	3	13·11	11·53†	−12·1†	13·55	+ 3·4
J.W.	1	2·82	3·16	+12·1	2·62	− 7·1
	2	6·28	6·71*	+ 6·8*	6·49	+ 3
	3	8·56	8·88	+ 3·7	8·57	+ 0·1
P.O.	1	5·65	5·77	+ 2·1	5·77	+ 2·1
	2	10·98	11·43	+ 4·1	11·05	+ 0·6
	3	15·93	16·97	+ 6·5	16·89	+ 6·0
E.H.	1	5·77	5·78	+ 0·2	5·51	− 4·5
	2	12·41	12·54	+ 1·0	12·71	+ 2·4
F.Y.‡	1	4·40	2·88	−34·5	1·67	−62·0
	2	8·07	6·46	−20·0	4·10	−49·2
P.S.‡	1	3·14	3·27	+ 4·1	4·36	+28·0
	2	6·29	6·94	+10·3	8·41	+33·7
	3	8·89	9·13	+ 2·7	10·06	+13·2
	4	11·83	11·90	+ 0·6	11·08	− 6·3

* Haemolysed specimen. † Haemolysed blank. ‡ Lipaemic specimens (subject not fasting).

TABLE III

STATISTICAL COMPARISON OF DIRECT AND EXTRACTION VALUES AND VARIATION BETWEEN EIGHT- AND 12-MIN. SAMPLES

Method	Variation from Extraction Value		Variation between 8- and 12-min. Samples	
	Mean	S.D. (%)	Mean	S.D. (%)
Extraction	—	—	1·3	1·1
Plasma				
All samples ..	−1·28	17·2	2·8	3·3
Clear samples ..	−0·4	6·5		
Serum				
All samples ..	−2·8	12·1	2·7	3·1
Clear samples ..	+0·4	5·8		

methods (Tables II and III). The mean difference between the eight- and 12-minute extracted samples was 1.3% (standard deviation 1.1%), further indication of the reliability of the method.

Direct Estimation in Plasma. — Excluding samples with haemolysis or lipaemia (patients F.Y. and P.S. had inadvertently eaten before the study), correlation (Table II, Fig. 1) is reasonably good, although the deviation of results and variation

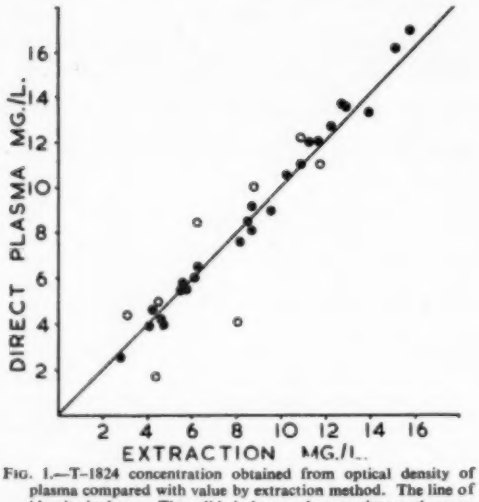

FIG. 1.—T–1824 concentration obtained from optical density of plasma compared with value by extraction method. The line of identity is shown. The solid circles are clear specimens, the open circles haemolysed or lipaemic samples.

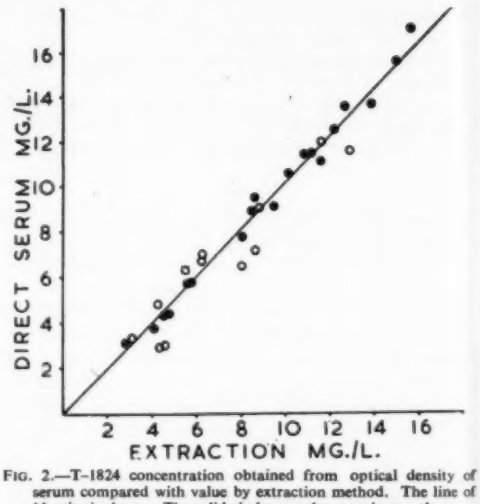

FIG. 2.—T–1824 concentration obtained from optical density of serum compared with value by extraction method. The line of identity is shown. The solid circles are clear specimens, the open circles haemolysed or lipaemic samples.

between eight- and 12-minute samples is greater than with the other methods (Table III).

Direct Estimation in Serum.—There is slightly better agreement (Table II, Fig. 2) with extraction values than was found with plasma; although haemolysis was more commonly encountered in handling serum, the effect of haemolysis and lipaemia was less marked (Table III).

Discussion

Figs. 1 and 2 show that even under ideal conditions in which the patient is fasting, and the plasma is not lipaemic or haemolysed, the direct method of estimating T–1824 concentration in plasma (or serum) has an error of approximately 6%. The occasional occurrence of lipaemia or haemolysis (approximately 10% of samples) will introduce considerably more error in the determination. The extraction technique, on the other hand, has an error of only 2.2% and is not influenced by the presence of haemolysis or lipaemia.

The accuracy of blood volume studies using a 10-minute sample and of indicator dilution curves recorded with an oximeter depends largely upon the accuracy of the measurement of T–1824 concentration in a single specimen. Use of the direct method of analysis in these procedures may introduce an error of considerable magnitude in the final results.

The extraction yield of 93.7% in the present study is not as high as the 97% reported by Allen (1951) and Bedwell *et al.* (1955) using similar methods. The reason for the lower yield is probably that we have worked at a lower temperature than have the other workers. The method has, however, given consistent results (known and duplicate solutions) in over 200 samples and has the added advantage of permitting simultaneous analysis of multiple samples.

Summary

A comparison was made of three methods for estimating the concentration of T–1824 in dye-circulation studies, namely, (*a*) an acetone-extraction method, (*b*) direct determination from optical density in plasma, and (*c*) direct determination from optical density in serum.

Direct estimation in the presence of lipaemia or haemolysis may be accompanied by considerable error.

The accuracy and reliability of the extraction method are discussed. In this laboratory it proved to be the most satisfactory for T–1824 analysis.

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PLASMA CHROMOGEN AND THE ENDOGENOUS CREATININE CLEARANCE

BY

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One of the troubles of using the endogenous creatinine clearance to measure the filtration rate is that plasma and serum contain chromogens, other than creatinine, which give a colour with the Jaffé reaction. The creatinine can, however, be isolated by adsorption on Fuller's earth (Gaebler and Keltch, 1928) and subsequently eluted with the alkaline picrate used for colour production (Borsook, 1935). This method has been used by a number of workers and its reliability established. Thus Hare and Hare (1949) found with radioactive carbon that recovery is complete, and Ralston (1955) with chromatography that no extraneous chromogen remains after treatment. But there is very little evidence as to the amounts of non-creatinine chromogen in the plasma or as to how neglect of its presence influences the creatinine clearance, and it is these points which will be considered here.

Method

Chromogens in Serum.—The extraneous chromogen is taken as the difference between total chromogen in untreated serum filtrates and true creatinine in the same filtrates treated with Fuller's earth. The total and extraneous chromogen are expressed as the amount of creatinine giving the same colour.

Reagents.—The following were used:

Standard creatinine solution, 0.5 g. in 1 litre $\frac{N}{10}$ HCl and dilutions with water to suitable strengths.

5% (w/v) sodium tungstate.

0.33 N. H_2SO_4.

Fuller's earth (B.D.H.), 10 g. in 100 ml. of water.

Oxalic acid, saturated solution (9.5 g. in 100 ml. of water).

0.75 N. NaOH (3%).

Picric acid, saturated solution.

Protein Precipitation.—Take two 2.5 ml. samples of serum in centrifuge tubes; to one (X) add 2.5 ml. of water and to the other (S) 2.5 ml. of 20 mg./l. creatinine solution and mix. To each add 2.5 ml. sodium tungstate and 2.5 ml. H_2SO_4. Mix and stand 10 minutes. Centrifuge and filter. When estimations are made both with and without adsorption double quantities must be used.

Total Chromogens.—To 5 ml. each of X, S, and water, add 3 ml. of a fresh mixture of equal parts of picric acid and NaOH. The colour of X is read against the H_2O blank and that of S against X, readings being made after 25 to 30 minutes at room temperature with a Spekker absorptiometer and blue-green filters, No. 603.

Creatinine.—To 5 ml. each of X, S, and water, in conical centrifuge tubes, add 1 ml. of Fuller's earth suspension and 0.5 ml. of oxalic acid. Stopper with plastic bungs and shake intermittently for 10 minutes. Remove bungs and wash them and the sides of the tube with a few drops of water. Centrifuge at 3,000 r.p.m. for 20 minutes. Decant the supernatant fluid and drain in reverse.

To the packed deposit add 8 ml. alkaline picrate solution made from 3 parts each of picric acid and NaOH and 10 parts of water. Stir, stopper, and shake intermittently for 10 minutes. Centrifuge for 10 minutes. The supernatant fluid is then poured off into the absorptiometer cuvettes and the colour of X read against the water blank, that of S against X. Readings are made at room temperature, the colour being fully developed at 30 minutes and remaining stable for several hours. Plastic bungs are better than rubber ones, since alkaline picrate removes a coloured compound from the latter if they are at all worn.

The concentrations of either total chromogen or creatinine are then given by the calculation:

$$\left(\frac{\text{Reading of X}}{\text{Reading of S}} \times 20\right) \text{ mg./l. creatinine.}$$

When the creatinine content of the serum is two to three times higher than normal, dilution is necessary. This is carried out after protein precipitation. The amount of standard creatinine added to the serum must then be increased so as to give a final concentration of 5 mg./l.

In addition to the internal standard, which is all that is needed for routine estimations, when total chromogen and true creatinine are being compared, standards in water of 2.5 to 10 mg./l. are used, both with and without treatment with Fuller's earth. The value of the serum is then read from the standard

curve in water and corrected for the colour depression which occurs in serum.

Thus if

Reading of serum = 0.15 = 15.6 mg./l.
Reading of serum + 5 mg./l. standard = 0.345
Expected reading of 15.6+5.0 mg./l. = 0.363
Corrected serum concentration

$$= 15.6 \times \frac{0.363}{0.345} = 16.4 \text{ mg./l.}$$

Creatinine Excretion Rates and Clearances.—The excretion rates are obtained from the means of three to 16 samples of urine. Creatinine is estimated in the urine without adsorption with Fuller's earth, since it has been shown that little if any extraneous chromogen is present (Hare and Hare, 1949; Owen, Iggo, Scandrett, and Stewart, 1954).

The mean minute excretion of creatinine divided by the total chromogen or creatinine in 1 ml. of urine gives the uncorrected creatinine clearance.

Results

The colours of alkaline creatinine picrate and alkaline picrate are influenced by the medium in which the creatinine is present and the temperature. In serum, creatinine gives less colour than in water (Roscoe, 1953) so that a standard in serum should be used, but the colour depression is less when the serum filtrates are treated with Fuller's earth than when they are untreated. Thus the colour given by creatinine added to 20 serum samples is 5.5% (S.D. 3.5) less than the expected without adsorption, only 1.6% (S.D. 2.3) less with adsorption. With adsorption there will, therefore, only be a small error if standards in water are used instead of the internal one, but these must be treated with Fuller's earth, because after treatment there is about 10% reduction in colour in all concentrations.

The colours of both alkaline picrate and alkaline creatinine picrate increase as the temperature rises and decrease again as it falls (Owen *et al.*, 1954). The increase in this laboratory is as much as 14% for alkaline picrate and 12% for alkaline creatinine picrate, between 15° and 25° C. It is therefore essential that the colours should be read with the blank, standards, and unknowns all at the same temperature. Here the readings were made at room temperature, since if, as has been recommended, the samples are brought to 25° C., they may cool unequally in cold weather, before all the readings are made.

Creatinine and Extraneous Chromogen in Serum.—Table I shows the true creatinine concen-

TABLE I
CREATININE AND EXTRANEOUS CHROMOGEN IN SERUM AND THE CREATININE EXCRETION AND CLEARANCE

Subject	Sex	Surface Area (sq. m.)	Plasma Creatinine (mg./l.)	Extraneous Chromogen (mg./l.)	Creatinine Excretion (μg./min./1·73 sq. m.)		Creatinine Clearance		
					M	F	Uncorrected (ml./min.)	Corrected (ml./min.)	Corrected (ml./min. 1.73 sq. m.)
1	F	1·76	8·0	4·0		770	65	97	96
2	F	1·31	8·4	1·6		400	30	36	47
3	M	1·93	9·0	4·5	1,030		85	128	115
4	M	1·85	9·7	1·95	1,040		96	115	107
5	M	1·71	10·0	4·0	1,410		100	140	142
6	M	1·97	10·1	3·7	1,100		91	124	109
7	M	1·64	10·3	1·9	860		67	79	83
8a	F	1·94	11·2	3·5		905	69	91	81
9	M	1·83	11·5	4·5	1,030		68	95	90
10	M	2·08	13·0	4·0	1,270		90	118	98
8b	F	—	13·1	2·4			57	67	—
11	M	1·56	13·5	2·3	920		52	61	68
12	M	1·79	13·7	2·05	1,220		80	92	89
13	M	1·62	14·6	2·1	1,220		69	78	83
14	M	—	16·25	3·25			62	74	
15	F	—	19·4	1·5			24	26	
16	M	—	19·9	3·0			40	46	
17	F	1·57	20·0	1·0		650	28	29	32
18	F	1·45	20·05	0		890	37	37	44
19	F	1·55	20·1	0·7		790	34	35	39
20a	M	2·02	20·5	4·1	1,065		50	61	52
21	M	—	22·4	2·3			43	47	
22	M	1·83	27·6	0·5	1,310		49	50	47
23	M	1·84	32·5	1·7	1,050		33	34	32
20b	M	2·02	46·5	1·0	1,005		25	25	21
24	M	—	47	1·5			22	23	
25	F	1·44	54	2·0		1,015	15	16	19
26	M	1·99	59	2·7	630		12	12	11
27	M	1·76	69	−1·0	845		13	13	12
28a	M	1·60	69	2·0	1,280		17	17	18
29	M	—	86·5	−3·0			14	14	
28b	M	1·57	140	0	925		6	6	6
30	F	—	150	0			2	2	

trations in 33 sera from 30 subjects and the extraneous chromogen concentrations. The extraneous chromogen varies from 0 to 4.5 mg./litre. It does not increase as the true creatinine concentration rises, but rather tends to be less. The mean concentration in 14 subjects with serum creatinines below 15.0 mg./l. (mean 11.15 mg./l.) is 3.0 mg./l., while in 10 subjects with serum creatinines of 15 to 33 mg./l. (mean 21.9 mg./l.) it is 1.8 mg./litre. These estimations were all carried out with 1 in 4 serum dilutions and may be compared; but with still higher serum creatinines, when higher dilutions are used, a constant amount of extraneous chromogen gives a decreased colour response, soon falling within the error of estimation, so that it is not possible to say whether it remains constant or decreases.

The proportion of the total chromogen provided by extraneous chromogen thus decreases as the creatinine rises. It is in this series, 21.4% in 14 samples with a mean creatinine concentration of 11.15 mg./l., 7.6% in 10 samples with a mean creatinine concentration of 21.9 mg./l., and 2.2% in five samples with a mean creatinine concentration of 55.1 mg./litre.

Creatinine Clearance.—Since the creatinine clearance is given by the minute excretion of creatinine divided by the plasma creatinine, if the total chromogen is used in its calculation it will be too low. The depression will be greater the higher the proportion of extraneous chromogen, and, since this is most in normal subjects with low plasma creatinines, it follows that the error will be greater in these, and will decrease as the plasma creatinine rises. The uncorrected and corrected clearances are shown in Table I and the difference is seen to be as much as 43 ml./min. in one case with a corrected clearance of 128 ml./min., while it is never more than 6 ml./min. when the corrected clearance is less than 50 ml./min. The corrected and uncorrected clearances are also compared in Fig. 1, where the decreasing difference at lower values is clearly seen. The curve shows the depression which will be produced if the creatinine excretion is 1,100 μg./min. and the extraneous chromogen 3.0 ml./litre.

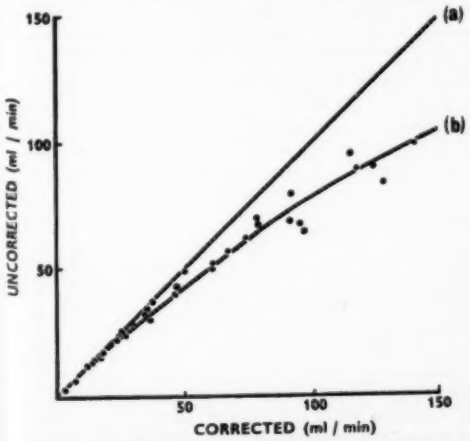

FIG. 1.—A comparison of the corrected and uncorrected values for creatinine clearance. The lines are those on which the points will lie (*a*) if there is no extraneous chromogen in plasma, (*b*) if the extraneous chromogen is always 3 mg./l. and the creatinine excretion rate 1,100 μg./minute.

The clearances also depend on the minute excretion of creatinine. This is given in Table I for those cases where the surface area is known. The excretion rate, corrected for surface area, is higher in men than in women, the means being 1,076 μg./min. in 17 men and 774 μg./min. in seven women. There are also large variations in rate within the male and female groups. There is no obvious falling off in excretion at lower filtration rates.

Discussion

Miller and Dubos (1937) estimated extraneous chromogen in serum, after destruction of the creatinine with an enzyme, and found it to be responsible for 10% of the total colour in normals, but for up to 50% in cases of nephritis with raised creatinine levels. In a later paper, however, Miller and Miller (1951), using both the enzymatic and the adsorption method, showed that, while 20% of the total chromogen was extraneous in normals, this decreased to 10% when the plasma creatinine was 10 times normal and to 5% when it was 20 times normal. Brod and Kotátko (1949) used adsorption with Lloyd's reagent and found 1.2–4.0 mg./l. (mean 2.8 mg./l.) of extraneous chromogen in sera from normals, but in renal failure this increased up to 135 mg./l. or 24% of the total chromogen. Hare and Hare (1949), with adsorption, again found approximately 20% of the total chromogen in normals to be extraneous, but only 10% or less in chronic renal insufficiency. Ralston (1955), with adsorption, found 2.8–5.0 mg./l. of extraneous chromogen in sera from 10 normals, or a mean of 36% of the total chromogen.

These results all agree in that in normals approximately 20% of the total chromogen is found to be extraneous and this has also been shown in the present work. When the true creatinine is increased the results of different workers vary widely, but the suggestion that the

extraneous chromogen accumulates and increases in the blood as kidney function fails is not on the whole supported, and this certainly does not occur in the cases studied in this paper where there is no increase or a fall.

Since the extraneous chromogen does not increase as the plasma creatinine rises and creatinine clearance falls, the error introduced by use of the total chromogen instead of the creatinine decreases, and when the plasma creatinine is more than twice normal, or the creatinine clearance less than 50 ml./min., it is usually negligible. On the other hand, with normal plasma creatinines the error may be very considerable, and, since the extraneous chromogen is by no means constant, it is not possible to correct by subtracting a mean value from the total chromogen, when this is the only estimate that has been made.

The clearance also depends directly on the creatinine excretion, and this varies widely. The lower excretion in females than males is related to the naturally lower clearances, but the variation found in one sex is sufficient to give a 50% difference in clearance, with the same plasma creatinine. This finding is not in agreement with that of Steinitz and Türkand (1940) and Effersøe (1957); the latter considered that the excretion is so constant for males or females respectively that the clearance can be calculated from the plasma value to within 20%. He used 24-hour clearances and ensured that the plasma creatinine was not changing, whereas here some clearances are only based on a few hourly samples and may not be representative. But in fact, in Case 2, with such a low excretion that, in spite of a low normal plasma concentration, the clearance is only 47 ml./min./1.73 sq.m., the excretion is the mean over 15 days during which the plasma creatinine did not alter.

A falling off in excretion rate in severe degrees of renal failure has been recorded by Steinitz and Türkand (1940). Here any such fall was obscured by an insufficiency of cases and the large variations even in normal subjects.

Summary

The total chromogenic material in serum filtrates, estimated by the Jaffé reaction, is compared with the true creatinine, separated from serum filtrates with Fuller's earth. Some practical points in the estimations are discussed.

The non-creatinine chromogen varies from 0 to 4.5 mg./l. creatinine equivalents in 33 sera. The amount does not increase and may even fall as the creatinine concentration rises.

If the total chromogen in plasma is used to calculate the endogenous creatinine clearance, this may be falsely depressed, the error being considerable in normal subjects, but decreasing as the filtration rate falls.

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SERUM HEXOSAMINE LEVELS IN HEALTH AND DISEASE*

BY

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Hexosamine is a normal component of serum mucoprotein and its level may be regarded as an index of total mucoprotein which includes α, β, and γ globulins, fibrinogen, "seroglycoid," and "seromucoid." Interest in seromucoids was greatly enhanced when it was found by West and Clarke (1938) and others that in acute and chronic infections, in malignancy, and in a number of endocrine disturbances, the hexosamine and inferentially the mucoid fractions increased above the normal range.

Raised serum hexosamine levels have been found in patients suffering from infection and disseminated malignant disease (West and Clarke, 1938; Weisbrod, 1950), in rheumatic fever (Rosenberg and Schloss, 1949; Kelley, 1952), in rheumatoid arthritis and asthma (Jiménez Díaz, Aguirre, and Arjona, 1953), and in pneumonia (Nilsson, 1937; Faber, 1948). The plasma hexosamine level also increases after extensive trauma (Schlamowitz, de Graff and Schubert, 1950; Boas and Peterman, 1953; West and Clarke, 1938).

In this paper, a systematic study has been made of serum hexosamine levels both in normal people and in a wide range of pathological conditions, in order to determine whether the presence of a high serum hexosamine level could be used as an indication of certain pathological states.

Method

Although numerous modifications of the Elson–Morgan (1933) method for the estimation of hexosamine have been described (Palmer, Smyth, and Meyer, 1937; Blix, 1948; Johnston, Ogston, and Stainer, 1951; Boas, 1953), none seemed entirely satisfactory for the handling of clinical material, where it is essential to be able to deal with large numbers of specimens without any significant loss in accuracy. Eventually a modification incorporating several methods was established and successfully employed as follows:

Solutions.—All solutions were prepared freshly on the day of use.

Ehrlich Reagent.—Recrystallized p-dimethylaminobenzaldehyde, 1.5 g., was dissolved in 75 ml. of 95% redistilled ethyl alcohol to which 25 ml. of concentrated HCl was then added and made up to a final volume of 500 ml. with 95% ethyl alcohol. This gives the optimum 5% concentration of acid recommended by Elson and Morgan (1933).

Acetylacetone Solution.—Concentrated acetylacetone (B.D.H.), 2 ml., was diluted with 0.75N Na_2CO_3 to 50 ml. This guarantees a final *p*H for the condensation reaction of approximately 9.7.

Standard Glucosamine Hydrochloride Solution.—A stock solution of glucosamine hydrochloride (200 mg. glucosamine in 100 ml. of water) was used to prepare solutions containing 20, 40, 60, 80, 100, 120, and 140 mg. glucosamine per ml.

Determination of Hexosamine.—One millilitre of 4N HCl was added to 1 ml. serum and the sample hydrolysed in a boiling water bath for six hours.

After cooling, the sample was neutralized with 4N NaOH to *p*H 6.5 using "universal" indicator paper, then transferred by means of a pipette to a 20 ml. standard flask, the tube being washed with several lots of distilled water. It was then made up to a final volume of 20 ml. and filtered through Whatman No. 42 filter paper.

To 1 ml. of filtrate in a special pyrex test tube (8 in. × ¾ in.) was added 1 ml. acetylacetone solution and the sides of the tube washed down with 1 ml. distilled water. The tube was stoppered with a long-necked sealed glass ampoule containing 2–3 ml. water. After heating 15 minutes in a boiling water bath, the rack of such tubes was transferred to an ice bath for 10 minutes.

Acetylacetone solution was also added to 1 ml. samples of suitable standard solutions and these tubes were heated at the same time as the unknowns. All the tubes were placed in the same metal rack so that they were simultaneously plunged and removed from the boiling water.

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† Working with the aid of a grant from the National Health and Medical Research Council of Australia.

When cool, the tubes were removed to a wooden rack and 10 ml. Ehrlich solution added to each and then shaken.

A "blank" was treated similarly.

After standing 30 minutes in the dark at room temperature, the colour intensity produced was read in a photoelectric colorimeter using a green filter. A standard curve was prepared and the concentration of the unknowns determined. This method does not differentiate between glucosamine and galactosamine: it gives the total hexosamine content of the hydrolysates.

Results

The failure of the ingestion of food to affect the serum hexosamine level in three normal individuals is shown in Table I.

TABLE I
EFFECT OF FOOD ON SERUM HEXOSAMINE LEVELS

Case	Hexosamine (mg. %)				
	Fasting	Time after High-protein-carbohydrate Meal			
		1 Hour	2 Hours	3 Hours	4 Hours
W. ..	110	119	104	108	115
D. ..	90	94	92	93	
W.S. ..	106	105	97	97	109

A high-protein, high-carbohydrate meal had no significant effect on serum hexosamine levels for a period of four hours following the meal. This had previously been shown in one individual by Shetlar, Foster, Kelly, and Everett (1948). According to Boas and Peterman (1953), fasting in rats does not lower the hexosamine level for the first 24 hours.

Variation of Serum Hexosamine Levels

The variation in the hexosamine level in an individual over a period of several weeks is only small, as is shown in Table II, which records serum hexosamine levels in six normal individuals at weekly intervals for several weeks.

TABLE II
WEEKLY SERUM HEXOSAMINE LEVELS IN NORMAL INDIVIDUALS

Case	Hexosamine (mg. %)				
	Time in Weeks				
	1	2	3	4	5
I.W. ..	100	96	100	96	
L.T. ..	96	95	96	96	
F.P. ..	96	92	96	94	
F.J. ..	86	87	86	88	84
K.G. ..	85	87	92	97	98
F.S. ..	100	106	101		

Serum Hexosamine Levels in Normal Subjects and in Various Pathological Conditions

Serum hexosamine determinations were carried out on 191 individuals and the results are expressed graphically in Fig. 1.

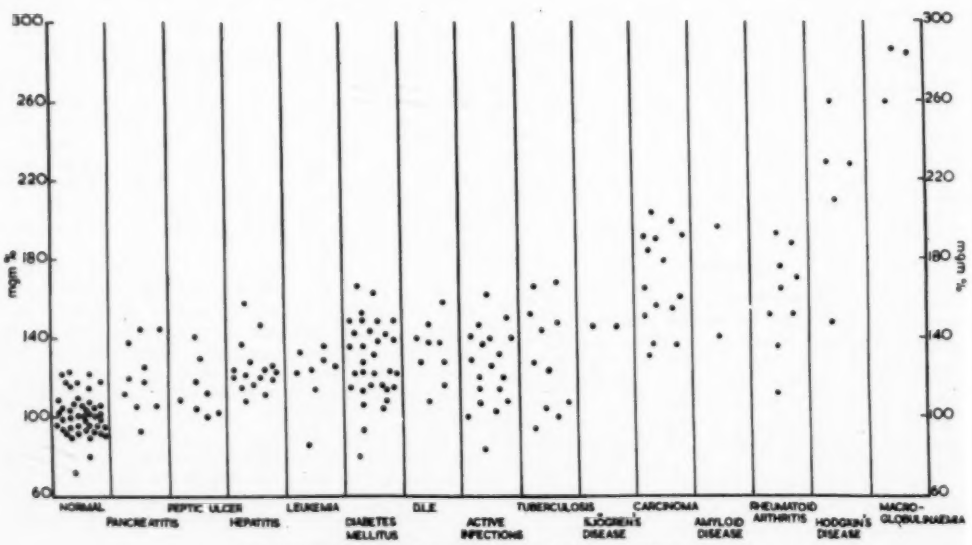

FIG. 1.—Serum hexosamine levels in normal and pathological conditions.

Normal Serum Hexosamine.—Normal serum hexosamine levels have been reported by a number of workers and cover a wide range (Table III).

TABLE III
HEXOSAMINE LEVELS IN NORMAL INDIVIDUALS

Author	No. of Cases	Hexosamine (mg. %) Range
Berkman *et al.* (1953)	15	84–116
Boas and Soffer (1951)	19	85–138
Faber (1948)	17	84–127
Jiménez Díaz *et al.* (1953)	10	75–125
Nilsson (1937)	19	63– 88
Palmer *et al.* (1937)	—	90–130
Rosenberg and Schloss (1949)	76	77–113
Shetlar *et al.* (1948)	28	62– 78
West and Clarke (1938)	21	76–110

In the present investigation blood was obtained from 45 normal individuals—staff members, nurses, and medical students. The mean average value was found to be 101.5 mg.% with a standard deviation of ±11.0 mg.% and the range 80–124 mg.% (±2 S.D.).

Pancreatitis.—Ten patients suffering from pancreatitis proved biochemically, and in some instances at operation, were found to have normal serum hexosamine levels. In three instances the values were above the upper range of normal, but not to any significant extent.

Peptic Ulcer.—All the eight peptic ulcer patients investigated had values in the normal range.

Liver Disease.—Of 16 patients with liver disease, two had acute infectious hepatitis, eight chronic infectious hepatitis, and five cirrhosis due to nutritional causes. Normal values were obtained in all but two patients—one with chronic infectious hepatitis who also showed lupus erythematosus cells in the blood (Mackay, Taft, and Cowling, 1956), and the other a patient with nutritional cirrhosis of the liver.

Using the turbidimetric method of Kunkel, Ahrens, and Eisenmenger (1948), the γ globulin content of the serum was determined in 15 of the patients with liver disease, but no close correlation could be shown between serum γ globulin and the hexosamine levels in these patients (Fig. 2).

Acute Infections.—In 20 patients with varying acute infections, there was a wide range of hexosamine levels from normal to 162 mg.% in a patient with pneumonia.

Tuberculosis.—Normal values were obtained in six of the 11 patients with active tuberculosis (eight pulmonary, two renal, and one tuberculous meningitis). It was found in the pulmonary cases

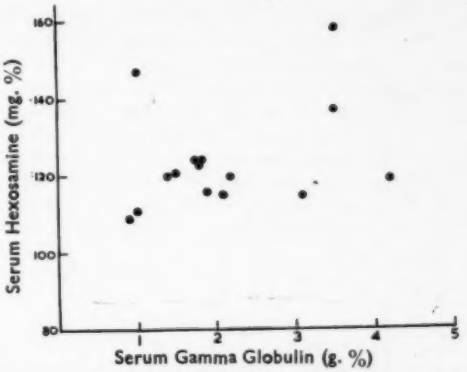

FIG. 2.—Chart showing the lack of correlation between serum hexosamine and gamma globulin levels in liver disease.

that the serum hexosamine increased with the severity of the disease. The highest value of 168 mg.% was obtained in a patient with extensive active pulmonary tuberculosis.

Systemic Lupus Erythematosus.—Increased values were obtained in six of the nine patients with lupus erythematosus tested; the remaining three were normal. The values ranged between 128 and 158 mg.% and are lower than those obtained by Boas and Soffer (1951) in this disease. As with the cases of liver disease, there was no close correlation between the hexosamine and γ globulin content of the serum in 17 determinations carried out on nine patients (Fig. 3). This is not in agreement with Boas and Reiner (1951), who claimed that the serum hexosamine in systemic lupus erythematosus was a reasonable index of the amount of circulating γ globulin in these patients.

Diabetes Mellitus.—The serum hexosamine level was determined in 32 patients suffering from diabetes mellitus of varying degrees of severity. Eighteen of the patients had values above the upper range of normal, the highest value being 166 mg.%. Raised values were present in both old and young diabetics, and irrespective of whether the control of the diabetes with insulin had been good or poor. This is in keeping with the findings of Taft, Finckh, and Joske (1954) which suggested that the lesions of the Kimmelstiel-Wilson syndrome would occur irrespective of whether there is efficient insulin therapy. Taft *et al.* (1954) used needle biopsies of the kidney in their studies.

In the present survey, 12 of the 26 diabetic patients in whom renal disease was not evident as

FIG. 3.—Chart showing the lack of correlation between serum hexosamine and gamma globulin levels in systemic lupus erythematosus.

yet had raised serum hexosamine. However, only four of the six patients with diabetic renal disease confirmed by renal biopsy had raised serum hexosamine values.

Leukaemia.—In six patients with chronic lymphatic leukaemia and in two with chronic myeloid leukaemia, there was only a slight rise in the hexosamine level. The highest values, 129, 133, and 136 mg.%, were in patients with chronic lymphatic leukaemia.

Carcinoma.—High serum hexosamine values were obtained in all but one of the 15 patients with carcinoma. In four instances the value was over 190 mg.%. The mean average value was 163 mg.%. Similar increases have been reported by other workers (Greenspan, Lehman, Graff, and Schoenbach, 1951; West and Clarke, 1938; Jiménez Díaz *et al.*, 1953; Faber, 1948).

Rheumatoid Arthritis.—Only one of the nine patients with rheumatoid arthritis had a serum hexosamine level within the normal range. The mean average value was 160 mg.% and the range 112–193 mg.%. This agrees with the previous findings of Jiménez Díaz *et al.* (1953) and Shetlar, Payne, Bullock, Patrick, Hellbaum, and Ishmael (1953).

Sjögren's Disease.—An identical value of 146 mg.% was obtained in two patients with Sjögren's disease, which is characterized by diminished lacrimal and oral secretions and arthritis.

Amyloid Disease.—Although amyloid disease is now a comparatively rare disease, serum was obtained from two cases in which the diagnosis was finally confirmed at post-mortem examination. The serum hexosamine values were elevated to 141 and 196 mg.% respectively.

Hodgkin's Disease.—Serum was obtained from five patients with Hodgkin's disease. Very high values were obtained in four of the cases, namely, 210, 228, 229, and 260 mg.%. The remaining patient had a raised value of 148 mg.%.

Macroglobulinaemia.—The highest values for serum hexosamine, 260, 284, and 286 mg.%, were obtained in three patients suffering from macroglobulinaemia, a disease first described in Sweden by Waldenström (1944) and characterized by an excessive production of serum globulins of an abnormally high molecular weight, detectable by ultracentrifugal analysis of serum. Macroglobulinaemia resembles multiple myeloma by reason of abnormal globulin production, but the histopathology is suggestive of a lymphocytic neoplasia, either chronic lymphatic leukaemia or lymphosarcoma. It has been suggested (Mackay, Taft, and Woods, 1957) that the basic process may, in fact, be a lymphosarcoma with macroglobulin synthesis, the result of a somatic mutation in the tumour cell line. Occasionally, however, macroglobulinaemia is associated with other types of neoplasm, and the condition may even exist in the absence of any apparent disease process (Laurell, Laurell and Waldenström, 1957).

Discussion

Hexosamine is a normal component of serum mucoprotein and its determination is an index of the total mucoprotein (Rimington, 1931, 1940; Mehl, Humphrey, and Winzler, 1949; Smith, Brown, Weimer, and Winzler, 1950; Schmid, 1953). It is also a component of various globulin fractions (Blix, Tiselius, and Svensson, 1941; Boas and Reiner, 1951; Boas, 1955; Werner, 1951). In the present investigation, the level for serum hexosamine was found to be between 80 and 124 mg.% in 45 normal individuals, and this agrees with values reported in the literature (Faber, 1948;

Palmer *et al.*, 1937; Rosenberg and Schloss, 1949; Berkman, Rifkin, and Ross, 1953; Jiménez Díaz *et al.*, 1953). The serum hexosamine level remained constant over a period of several weeks, and was not significantly altered by food.

Pancreatitis, peptic ulcer, and liver disease had no effect on the serum hexosamine level which was, however, significantly increased in those diseases associated with bacterial infection and tissue proliferation. Thus moderately raised values were obtained in patients with pneumonia and tuberculosis and the height of the increase corresponded to the severity of the disease. Increased values were also obtained in patients with systemic lupus erythematosus, but no correlation could be found in these patients between the γ globulin and serum hexosamine level as reported by Boas and Reiner (1951). The same lack of correlation with γ globulin was found in the patients with liver disease.

Raised serum hexosamine values were obtained in approximately 50% of patients with diabetes mellitus. The high values were in patients with renal disease and in 12 of the 28 patients without renal disease. The nature of diabetic renal disease remains obscure. Abnormal protein material, which may line endothelial surfaces and infiltrate blood vessel walls, is deposited in the kidney. The staining properties of this material suggest that it contains protein, polysaccharide, and some lipid. It would seem that in some cases of diabetes mellitus there is an underlying metabolic defect involving hexosamine metabolism which produces an increase in mucoprotein in the blood and its deposition in the kidney. It is not yet known whether the deposition comes first and causes the renal damage or whether there is a pre-existing renal damage. Raised hexosamine values were obtained in both young and old diabetics and irrespective of whether the control of the diabetes with insulin and diet had been good or poor. Similar findings were obtained by Taft *et al.* (1954) in their needle biopsy study of renal lesions in the Kimmelstiel–Wilson syndrome. Isotope studies in animals indicated that the mucosubstances of the body are in a continuous state of turnover (Schiller, Mathews, Jefferson, Ludowieg, and Dorfman, 1954; Schiller and Dorfman, 1955).

The biosynthesis of mucosubstances has considerable importance in pathological conditions, particularly those where extensive proliferation of cells occurs. Human and animal synovial membrane grown in tissue culture produced hyaluronic acid (Kling, Levine, and Wise, 1955; Grossfeld, Meyer, and Godman, 1955), a hexosamine-containing mucopolysaccharide which is the main constituent of the ground substance of connective tissue (Meyer and Rapport, 1951). Raised serum hexosamine therefore can be regarded as a non-specific index of tissue destruction, or of proliferation and repair. In the present study high values for serum hexosamine were obtained in patients with Hodgkin's disease and carcinoma, where proliferation and breakdown of tissue existed with probable shedding of the breakdown products into the tissue spaces and absorption into the blood stream. Thus the determination of the serum hexosamine level is a valuable aid in the differential diagnosis of Hodgkin's disease from chronic infections where values are not very high.

Increased synthesis of mucopolysaccharides in the ground substance of connective tissue in rheumatoid arthritis could account for the high serum hexosamine values obtained in the patients with this disease and for the pain and stiffness of the joints. As cortisone will inhibit connective tissue formation (Layton, 1951a, 1951b), this may be responsible for the improvement in patients with rheumatoid arthritis when treated with this compound.

The highest values for serum hexosamine obtained in this study were in patients suffering from macroglobulinaemia as opposed to normal values in leukaemia. In macroglobulinaemia there is a synthesis of abnormal serum globulins, macroglobulins, which are probably elaborated by the reticulo-endothelial plasmacyte system (Mackay, Eriksen, Motulsky, and Volwiler, 1956).

Summary

(1) The determination of the hexosamine level of serum is an index of its total mucoprotein content.

(2) The range for serum hexosamine in 45 normal individuals was found to be 80–124 mg.% with a mean average value of 101.5 mg.% $\pm$11 mg.%.

(3) Ingestion of food did not alter serum hexosamine levels, which remained constant over a period of weeks as shown by random sampling in normal controls.

(4) The serum hexosamine level was normal in cases of peptic ulcer, pancreatitis, liver disease, and leukaemia.

(5) It was moderately raised in active infections such as tuberculosis, pneumonia, and bacterial endocarditis.

(6) Markedly increased values were obtained in systemic lupus erythematosus, rheumatoid arthritis, carcinoma, amyloid disease, Hodgkin's disease, and macroglobulinaemia.

(7) The high level of serum hexosamine in Hodgkin's disease and macroglobulinaemia can be used as an aid in the diagnosis of these diseases.

(8) No correlation could be found between serum hexosamine and γ globulin levels in patients with hepatitis or systemic lupus erythematosus.

(9) In diabetes mellitus more than half of the patients showed some increase in serum hexosamine levels. This rise could not be correlated with the efficiency of the control of the hyperglycaemia by insulin.

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NORMAL FAECAL PORPHYRIN VALUES IN BABIES

BY

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Normal faeces contain both coproporphyrin and protoporphyrin in very small quantities. These arise in part as by-products from haem synthesis which are excreted via the bile into the gut, in part from degradation of ingested haem, and possibly also in part by bacterial synthesis in the intestine. In adults normal values (Barnes, 1957) are: coproporphyrin, 0–15 μg. per g. dry weight; and protoporphyrin, 0–45 μg. per g. dry weight.

The purpose of this investigation was to establish normal values in babies from birth up to 1 year of age. Only one reference in the literature has been found to porphyrin determinations in babies and children (Fokina, 1947), but, whereas Fokina has estimated average faecal elimination values per 24 hours, these values are expressed as micrograms per gramme dry weight and therefore the two investigations are not strictly comparable.

Material

Random specimens of stool were collected from babies on ordinary hospital diet, with no history of porphyrinuria and suffering from diseases or conditions not thought to influence porphyrin metabolism. Determinations were repeated several times during the patients' stay in hospital. The specimens of stool were yellow to khaki in colour; none was dark brown or black.

The age and sex distribution of the babies are shown in Table I.

TABLE I
AGES AND SEX OF BABIES INVESTIGATED

Age	No. of Patients	Male	Female
0–6 months	11	5	6
7–12 ,, ...	9	3	6

Method

Rimington's method (Barnes, 1956) of partitioning the porphyrins and his quantitative analysis was used.

Extraction of Coproporphyrin.—The weighed faeces were dried in an oven at 105° to 110° C. One gramme of the specimen was treated with acetic acid and extracted with ether. This extraction was repeated until no orange-red fluorescence could be detected. The pooled extract was washed with saturated sodium acetate and 0.005% iodine. The ether solution was then treated as follows. For the extraction of coproporphyrin, shake as above using successive amounts of 0.1N hydrochloric acid until no further red fluorescent material is removed.

Extraction of Protoporphyrins (Non-coproporphyrins).—This was carried out similarly with 1.5N hydrochloric acid. The two extracts were made up to convenient volumes and read on a Beckman spectrophotometer at 430, 380 mμ and at their peak values. The results were expressed in micrograms per gramme dry weight of faeces.

Results

The findings in both age groups are shown in Fig. 1. It will be seen that except in two cases there was no wide variation in results, and amounts of both coproporphyrin and protoporphyrin were lower than those in normal adults (Barnes, 1956). This lowered output can be accounted for by the meatless diet consumed by the infants. In two cases, however, protoporphyrin results much higher than the others were obtained. Both babies were suffering from infection, one (aged 9 months) from infected burns and the other (aged 12 months) from periorbital cellulitis.

During the course of this investigation one child (aged 2 months) contracted acute laryngo-tracheobronchitis; whilst the coproporphyrin remained unchanged (1.0 and 1.1 μg. per g. dry weight) the protoporphyrin rose sharply from 3.0 to 32.5 μg. per g. dry weight. With the exception of these three cases, estimations repeated on several occasions gave similar results. It can be concluded that the range for normal values for both coproporphyrin and protoporphyrin in infants is 0 to 10 μg. per g. dry weight.

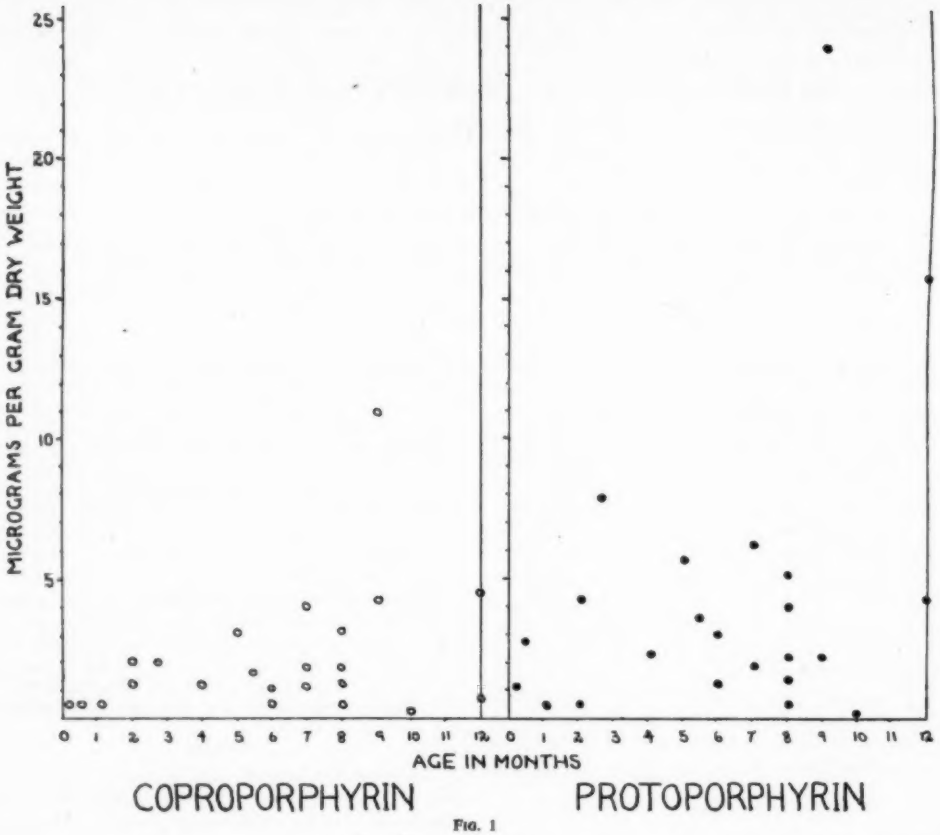

FIG. 1

Summary

Coproporphyrin and protoporphyrin levels in babies were determined in order to establish normal values.

We wish to thank the Director of the Institute for his interest and for facilities for carrying out this work, Dr. H. D. Barnes for his help and encouragement, and Mrs. N. Stevens for her technical assistance.

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THE SENSITIVITY OF VARIOUS BACTERIA TO ANTIBIOTICS DURING THE YEARS 1951 TO 1956

BY

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Since the beginning of 1951 a record has been kept of the sensitivity to available antibiotics of most common bacterial pathogens isolated from routine material in the clinical laboratory of a large provincial general hospital. The most commonly tested organisms included *Staphylococcus pyogenes aureus (Staph. aureus), Escherichia coli (E. coli), Pseudomonas pyocyanea,* and members of the *Proteus* and *Klebsiella* group of organisms. An analysis follows of the results of the sensitivity tests carried out during the six years from 1951 to 1956 on these bacterial species.

Technique

Qualitative methods form the basis of this study. At the outset filter paper strip methods were employed. During the course of time, however, this practice was abandoned in favour of the dried filter paper disc technique (Thompson, 1950; Fairbrother, Martyn, and Parker, 1951). When standard antibiotic test tablets (Evans's "sentests") became available for most of the commoner drugs these were substituted for filter paper discs, since it was found that far more consistent and reliable results were thus obtained. These tablets contain the following concentrations of the different antibiotics:

Penicillin	2.5	Oxford units per tablet
Streptomycin	20	μg. " "
Aureomycin	100	" " "
Terramycin	100	" " "
Tetracycline	100	" " "
Chloramphenicol	40	" " "
Erythromycin	10	" " "

In the case of polymyxin B dried paper discs each containing 150 μg. were used. An organism was judged sensitive if a zone of inhibition of 10 mm. radius or more surrounded the tablet or paper disc. Media employed consisted mainly of blood agar for *Staph. aureus* and MacConkey's bile salt agar for the Gram-negative bacteria. Results were read after 15 to 18 hours' incubation. For sulphonamide sensitivity of *E. coli* the method of Jewell and Pearmain (1954) was adopted.

Staphylococcus Aureus

Altogether 1,565 strains were examined. These were derived from the following sources:

Wounds, abscesses, septic skin lesions	671
Eye swabs	429
Sputum	124
Urinary infections	121
Vaginal swabs	75
Nose and throat	66
Blood culture	26
Elsewhere	53

As Barber (1947), Forbes (1949), and others have pointed out, most penicillin-resistant strains originate in hospital, and the same probably holds true for resistance to other antibiotics. Unfortunately, it was not possible in such a large series extending over several years to be certain in every case whether infection originated in hospital or outside. However, the majority of specimens was taken from in-patients, and it can be assumed that a high proportion of these were in fact suffering from hospital infections. In any case, the proportion of in-patients to out-patients from whom bacteriological specimens were taken has remained the same between 1951 and 1956. A valid comparison is therefore possible between the figures for each year.

Results.—The results of antibiotic sensitivities of *Staph. aureus* are summarized in Table I, and the more striking changes in Fig. 1. Most of the strains resistant to several antibiotics were also penicillin-resistant, as already observed by Clarke, Dalgleish, and Gillespie (1952). There has been a marked increase since 1955 of strains resistant to three or more antibiotics, as shown by the following figures:

1951	6 strains
1952	6 "
1953	19 "
1954	14 "
1955	42 "
1956	62 "

A preliminary survey of results for the first four months of 1957 suggests that there will probably be a further sharp rise during the current year of staphylococcal strains resistant to many or most of

TABLE I
SENSITIVITY OF *STAPHYLOCOCCUS AUREUS* TO ANTIBIOTICS

Year	Penicillin		Erythromycin		Streptomycin		Aureomycin		Terramycin		Tetracycline		Chloramphenicol	
	Total No.	% Resistant	Total No.	% Resistant	Total No.	% Resistant	Total No.	% Resistant	Total No.	% Resistant	Total No.	% Resistant	Total No.	% Resistant
1951	157	64			153	17	157	6					128	4
1952	232	68			232	16	232	4					232	5
1953	288	73			289	18	280	8	280	8			276	5
1954	285	59	242	1	280	9	269	6	278	6			282	5
1955	279	72	280	2	283	20	284	15	276	18	156	15	286	6
1956	302	68	294	1	302	33	300	24	302	22	302	23	303	10
Total 1951 to 1956	1,543	68	816	1	1,539	19	1,522	11	1,136	14	458	21	1,507	6

the common antibiotics. Three strains of this type during the latter half of 1956 caused fulminating entero-colitis and two of these cases were fatal.

In recent months all antibiotic-resistant staphylococci have also been tested against novobiocin, oleandomycin, and spiramycin, but no resistance to any of these newer antibiotics has as yet been encountered. According to these figures, penicillin-resistance, though fluctuating between 59 and 73%, has not shown a steady rise as in earlier years. Thus Barber and Rozwadowska-Dowzenko (1948) found an increase from 14% in 1946 to 59% in 1948. But already in 1953 Needham and Nichols noted that penicillin-resistance among staphylococci had not substantially increased between 1948 and 1952. Penicillin-resistance at different times has been quoted as 42% (Fairbrother *et al.*, 1951), 10% (MacFarlane, 1951), 60–80% (Kenney, Johnson, and Tatz, 1953), 43–70% (Weil and Stempel, 1953), 70% (Kirby and Ahern, 1953), and 82% (Rantz and Rantz, 1956).

Resistance to streptomycin in the present series has, however, increased since 1955, and there is a well-marked rise in the resistance to the tetracyclines during the last two years of this survey. Chloramphenicol has shown the same trend, but to a much lesser degree. Many authors, especially in America, reported similar changes several years ago (Needham and Nichols, 1953; Weil and Stempel, 1953; Kenney *et al.*, 1953). A recent

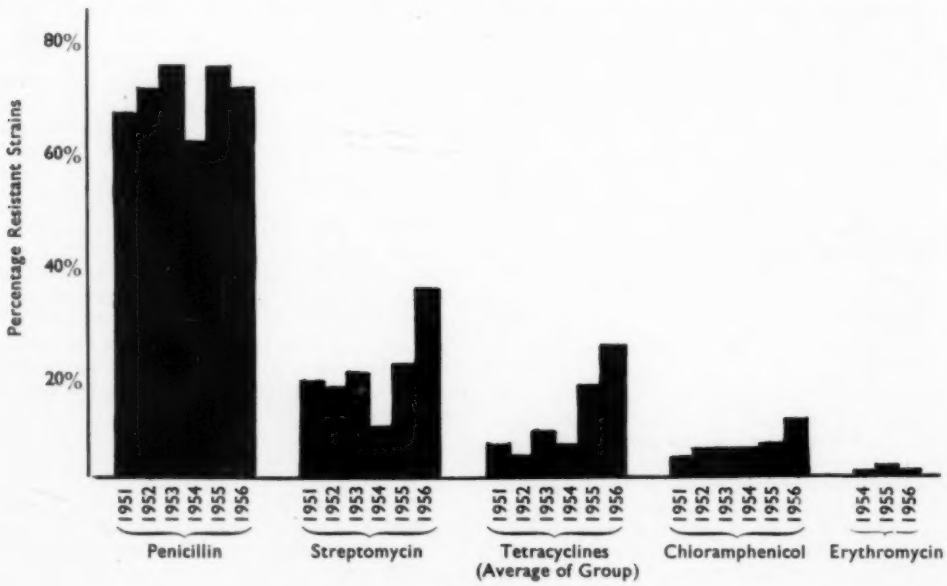

FIG. 1

review by Rantz and Rantz (1956) showed over half their strains of *Staph. aureus* to be resistant to streptomycin and tetracycline, while less than 6% were chloramphenicol resistant.

Although erythromycin has been freely available for nearly four years, resistance to this drug has not as yet presented a serious problem in this hospital, although Rantz and Rantz (1956) already reported a 6.6% resistance rate. One of the reasons for the continued efficacy of erythromycin in staphylococcal infections may have been its restricted use by the hospital medical staff. A conscious effort was made to use this antibiotic only in cases infected with staphylococci resistant to other drugs and in staphylococcal infections of young children or very seriously ill patients. Since the newer antibiotics such as novobiocin, oleandomycin, and spiramycin can now be obtained it might be tempting to use erythromycin more freely. However, there is already some evidence (*Lancet*, 1957) that resistance to at least two of the new drugs may result in cross-resistance to erythromycin.

Escherichia Coli

The total number of strains examined was 2,827. They were derived from the following sources:

Urinary infections	2,062
Wounds, abscesses, ulcers ..	398
Vaginal swabs	167
Elsewhere	200

Tetracycline sensitivity tests were not performed regularly until 1954 and the figures for 1951–53 have therefore been omitted.

Results.—These are set out in Table II. In addition, 537 strains were tested for sensitivity to three sulphonamides (sulphathiazole, sulphadiazine, and sulphadimidine). Of these, 17% were resistant to all three drugs, and 63% to one or two.

It is evident that in this series there has been no significant increase in the resistance of *E. coli* to the commoner antibiotics. The slightly higher rate of resistance to aureomycin as compared with the other two tetracyclines may at first sight appear rather puzzling. However, partial drug resistance of organisms, especially to the tetracyclines, is not uncommon. By definition an organism is classed here as sensitive to a drug if the zone of inhibition was 10 mm. radius or more. Some strains, however, show a somewhat smaller zone of inhibition and are therefore listed as resistant strains. Such a division is, of course, an arbitrary one and may explain this apparent anomaly.

If sensitivity *in vitro* is any guide, chloramphenicol is the most, and streptomycin the least, effective of the antibiotics under review. These findings are consistent with earlier work by Fairbrother *et al.* (1951) and May and Morley (1952). Most American workers record a significantly higher resistance rate. Thus Horton and Knight (1955) found 27–33% of urinary strains of *E. coli* resistant to chloramphenicol and up to 88% resistant to the tetracyclines and streptomycin; earlier, in 1953, Weil and Stempel recorded 10–17% resistant strains in the case of chloramphenicol, up to 54% for the tetracyclines, and 45–64% for dihydrostreptomycin. In the series published by Rantz and Rantz (1956) about 40% of all *E. coli* strains were resistant to streptomycin and tetracycline, while 34% were chloramphenicol resistant. They also found an increase in the rate of antibiotic resistance over a five-year period. Most workers agree with Perry (1955) that chloramphenicol is still the most effective antibiotic against *E. coli*.

Proteus.—Altogether 463 strains were examined, most of which were *P. vulgaris*, although a small number of specimens yielded *P. mirabilis* and *P. rettgeri*. They were derived as follows:

Urinary infections	303
Wounds, abscesses, ulcers	97
Elsewhere	63

The number of *Proteus* strains isolated from sites where they were considered pathogenic has steadily increased since 1951. Although the

TABLE II

SENSITIVITY OF *ESCHERICHIA COLI* TO ANTIBIOTICS

Year	Streptomycin		Aureomycin		Terramycin		Tetracycline		Chloramphenicol	
	Total No.	% Resistant	Total No.	% Resistant	Total No.	% Resistant	Total No.	% Resistant	Total No.	% Resistant
1951	342	18							322	7
1952	395	8							394	4
1953	433	8							423	2
1954	510	17	449	13	500	8			507	5
1955	466	13	459	9	464	3	399	9	468	2
1956	430	13	431	6	427	3	421	3	428	2
Total 1951 to 1956 ..	2,576	13	1,339	9	1,391	5	820	6	2,542	3

TABLE III
SENSITIVITY OF *PROTEUS* TO ANTIBIOTICS

Year	Streptomycin		Aureomycin		Terramycin		Tetracycline		Chloramphenicol	
	Total No.	% Resistant	Total No.	% Resistant	Total No.	% Resistant	Total No.	% Resistant	Total No.	% Resistant
1951	29	28							30	27
1952	36	28							34	21
1953	42	31							43	28
1954	76	36	73	82	75	85			77	12
1955	119	42	124	90	121	85	115	87	119	24
1956	122	43	122	94	122	83	122	82	122	37
Total 1951 to 1956 ..	424	38	319	90	318	84	237	84	425	26

reason for this higher incidence is not altogether clear, the popularity of the tetracyclines may have favoured the survival of this type of organism in places like hospital wards where they may remain as endemic foci of hospital infection.

Results.—Results of sensitivity tests are shown in Table III. From these it is clear that only chloramphenicol and, to a lesser degree, streptomycin were effective against this group of organisms. There has, however, been a slight but steadily increasing drug resistance in the case of streptomycin and, in 1956, also to chloramphenicol. Only a minority of strains were inhibited by the tetracyclines. Nitrofurantoin has also been tested for a number of years, but only on rare occasions has it been found effective against strains resistant to the common antibiotics. Most organisms exhibiting universal drug resistance were isolated from cases of long-standing urinary infections in elderly males with prostatic disease, all of whom had received previous drug therapy. Neomycin has only recently been tried, but the number of tests are as yet too small to warrant publication.

British reviews (Fairbrother *et al.*, 1951; May and Morley, 1952) produced similar results, whereas more recent American papers (Weil and Stempel, 1953; Horton and Knight, 1955; and Rantz and Rantz, 1956) found a rather higher rate of drug resistance. Unlike Poole (1954) and Potee, Wright, and Finland (1954) no constant drug sensitivity patterns for the different biochemical types of *Proteus* were found.

Pseudomonas Pyocyanea

A total of 195 strains are included. Their sources were as follows:

Urinary infections	95
Wounds, ulcers, etc.	54
Ears	14
Elsewhere	32

Results.—Results of sensitivity tests are seen in Table IV. A fluctuating but uniformly high rate of drug resistance is evident from the figures in the present series. Unlike the *Proteus* group, *Ps. pyocyanea* is resistant to chloramphenicol even more often than to the three tetracyclines and streptomycin. The only antibiotic which was effective in inhibiting the growth of about four out of every five strains of *Ps. pyocyanea* was polymyxin B. The present series thus confirms the findings by Wright, Potee, and Finland (1954), but contrasts with those of Yow and Townsend (1953), who claim that 99% of strains were sensitive to polymyxin and 88% to terramycin.

Klebsiella

The broader definition by Kauffmann (1951) has been adopted for purposes of classification in the present study. In this group, therefore, are included all mucoid lactose- and inositol-fermenting strains with a negative indole and a positive

TABLE IV
SENSITIVITY OF *PS. PYOCYANEA* TO ANTIBIOTICS

Year	Streptomycin		Aureomycin		Terramycin		Tetracycline		Chloramphenicol		Polymyxin B	
	Total No.	% Resistant	Total No.	% Resistant	Total No.	% Resistant	Total No.	% Resistant	Total No.	% Resistant	Total No.	% Resistant
1951	34	79							35	71		
1952	34	65							33	91	22	5
1953	23	70							23	91	24	33
1954	27	78	28	71	27	85			28	89	27	30
1955	24	58	24	88	24	67	24	79	24	92	22	18
1956	38	84	37	92	37	76	36	72	37	78	28	14
Total 1951 to 1956 ..	180	73	89	84	88	76	60	75	180	84	123	20

TABLE V
SENSITIVITY OF *KLEBSIELLA* TO ANTIBIOTICS

Year	Streptomycin		Aureomycin		Terramycin		Tetracycline		Chloramphenicol	
	Total No.	% Resistant	Total No.	% Resistant	Total No.	% Resistant	Total No.	% Resistant	Total No.	% Resistant
1951	25	44							32	22
1952	39	59							39	10
1953	35	57							35	17
1954	13	39	12	42	10	10			13	8
1955	21	62	20	20	20	0	19	16	20	0
1956	46	35	39	13	38	11	37	8	38	8
Total 1951 to 1956 ..	179	49	71	20	68	7	56	11	177	12

Voges Proskauer reaction. The 191 strains were derived from the following sources:

Urinary infections	128
Sputum	45
Wounds	13
Elsewhere	5

Results.—Results of sensitivity tests, as seen in Table V, show that chloramphenicol and the tetracyclines are about equally effective and that there is no sign as yet of increasing rates of resistance. For reasons not understood, chloramphenicol-resistant strains appear to have been diminishing in recent years rather than the reverse. Many of the urinary strains were streptomycin-resistant, whereas most strains of *K. pneumoniae* were sensitive to this antibiotic. The figures in the present series are in marked contrast to those published by Horton and Knight (1955) and Rantz and Rantz (1956) for *Aerobacter aerogenes*, since most strains in both American series were resistant to the common antibiotics.

Summary and Conclusions

The sensitivity of 5,241 common bacterial pathogens from 1951 to 1956 is reviewed.

The number of strains of *Staphylococcus aureus* resistant to penicillin during this period has remained fairly constant. Most strains are still sensitive to erythromycin and chloramphenicol, whereas the incidence of strains resistant to streptomycin and the tetracyclines has risen in recent years. The number of staphylococci showing resistance to three or more antibiotics has increased considerably.

There has been no significant rise in the rate of drug resistance of *Escherichia coli* to the commoner antibiotics. Chloramphenicol appears to be the most effective drug at present.

Members of the *Proteus* group are sensitive more often to chloramphenicol and streptomycin than to the tetracyclines, but the incidence of strains resistant to the first two is mounting.

Pseudomonas pyocyanea is usually resistant to most antibiotics with the exception of polymyxin B.

Of the *Klebsiella* group of organisms, most of the urinary strains showed marked resistance to streptomycin. Strains resistant to chloramphenicol and the tetracyclines were less frequent.

The incidence of antibiotic resistance in this series appears to be generally lower than in recent American surveys.

Our thanks are due to Mrs. Margaret Clowes, Mr. A. C. Key, F.I.M.L.T., Mr. P. H. Johnson, A.I.M.L.T., and Mr. R. Astbury, F.I.M.L.T., for technical assistance.

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TECHNICAL METHODS

A Simple and Safe Method for Determining the Sensitivity of Tubercle Bacilli to Antituberculous Agents

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(RECEIVED FOR PUBLICATION SEPTEMBER 9, 1957)

Chemotherapy of tuberculosis, to be effective, demands adequate laboratory control of antituberculous drugs. It is, therefore, important to establish a convenient, reliable, and rapid method of handling large numbers of sensitivity tests *in vitro*.

The method to be described employs a composite medium for primary isolation of the tubercle bacillus. This medium consists of a Löwenstein-Jensen (L.J.) slope incorporating in the bottom of the tube 2 ml. of modified Besredka (1921) liquid egg yolk medium.

Modified Besredka Medium

The yolks of two eggs are beaten up and added to 200 ml. of distilled water. It is important that all egg white be removed. After thorough shaking, the mixture is clarified by adding to it 1% NaOH (75 to 80 ml. should be sufficient). The clarified mixture is then filtered through Chardin paper and the volume of the filtrate made up to 600 ml. with distilled water. The *p*H is adjusted to 8.0 with N/20 HCl, after which the medium is bottled in 100 ml. amounts. Sterilization is carried out by tyndallization. Just before the final steaming, 0.5 ml. of 2% malachite green is added to each 100 ml. amount. For use, the medium is distributed aseptically in 2 ml. amounts into the bottom of L.J. slopes.

Procedure

Specimens after decontamination and concentration are inoculated into Löwenstein-Jensen-Besredka (L.J.B.) slopes, which are incubated horizontally at 37° C. for one week. They are then set upright and incubation continued for six weeks before being discarded as negative. Growth is usually apparent on the surface of the medium in 11 to 21 days. Growth characteristics are identical with those obtained on standard L.J. slopes.

Sensitivity Testing

The fluid portion of the primary culture is used as the inoculum for L.J. slopes in which are incorporated the appropriate concentrations of streptomycin, isonicotinic acid hydrazide (isoniazid), and *p*-aminosalicylic acid (P.A.S.). An L.J. slope with no added antituberculous agent is inoculated for each strain under test as a growth control.

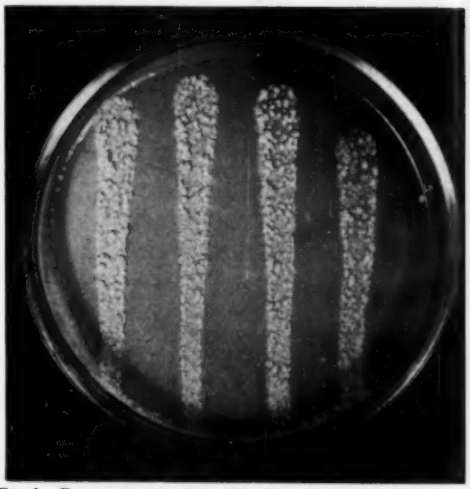

FIG. 1.—Four consecutive drops of the Besredka portion of a primary L.J.B. culture of tubercle bacilli have been delivered from a pasteur pipette and allowed to run down the surface of L.J. medium. The uniformity of inoculum attainable by this means is indicated by the identical growths resulting after 26 days' incubation at 37° C.

For inoculation of test media, one drop of the Besredka growth from the primary culture is allowed to run down the surface of each of the test and control slopes, using a Pasteur pipette fitted with a rubber teat. The tests are incubated at 37° C. for 21 to 28 days, after which results are read. A sensitivity test is accepted as readable only if the growth control tube contains over 20 colonies or a confluent growth. This ensures that an adequate inoculum has been made into each tube under test because, as shown in Fig. 1, consecutive drops of Besredka primary culture from the same pipette produce identical growths on fresh L.J. medium.

Discussion

Determination of the sensitivity of strains of tubercle bacilli to antituberculous drugs, especially where large numbers of specimens are handled, can be laborious, time-consuming, and dangerous to the operator. The method described has been adopted in order to minimize these drawbacks. It is essentially a solid medium method of sensitivity testing, and is, therefore, suitable for use with streptomycin (Stewart,

1955), isoniazid, and P.A.S. (Medical Research Council, 1953). The culture media and techniques employed are within the resources of most bacteriological laboratories. Primary growths of tubercle bacilli on L.J.B. medium are readily recognizable and this medium has been found to be as satisfactory as L.J. medium for isolating the organisms from sputum concentrates. The fluid portion of a primary culture, when used in the manner described, provides, for all practical purposes, uniform inocula for the test and control slopes of sensitivity tests, thereby obviating recourse to a second subculture with its attendant delay and potential inaccuracies (Mitchison, 1953). Risks to technicians from mechanical emulsification of tubercle bacilli are also avoided. The simplicity of the method enables large numbers of sensitivity tests to be performed rapidly (approximately 24 per technician per hour) so that the extent to which different concentrations of test substances are used need virtually be limited only by available incubator space. The method has proved itself convenient to use in a general laboratory dealing with some 3,000 sensitivity tests per annum. It has provided results which clinical colleagues have found to be consistent and reliable.

Summary

A simple, safe, and efficient method is described for determining the sensitivity of tubercle bacilli to standard antituberculous agents.

I wish to thank Dr. A. J. O'Hea, Department of Bacteriology, University of Glasgow, for advice and criticism.

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Paper Electrophoresis of Urinary Porphyrins

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(RECEIVED FOR PUBLICATION MAY 24, 1957)

A simple method is described for the isolation and the determination of the different porphyrins excreted in urine in cases of porphyria.

Twenty-four hours' urine is collected with toluene as preservative and 500 ml. is taken for the extraction of the porphyrins. The urine is acidified with glacial acetic acid to *p*H 4.0 and left for 24 hours covered with a layer of toluene for the separation of the porphyrins. The porphyrins separate as a brown precipitate, which partly floats in the urine. The precipitation of porphyrin is not complete, but a small amount which precipitates much more slowly was found by electrophoresis to have the same composition as the mixture which comes down in the first 24 hours' standing. The urine is filtered through Whatman No. 42 filter paper. The precipitate on the filter paper is washed with distilled water till the washings are neutral to litmus. Barbitone buffer (10.3 g. of sodium diethyl barbituric acid and 1.84 g. of diethyl barbituric acid in 1 litre of water) is prepared and a minimum volume of this buffer (about 5 to 8 ml.) is poured on the filter paper over and over again to dissolve the porphyrins. The precipitate dissolves completely giving a deep red solution. This solution is used for the separation of the porphyrins by paper electrophoresis.

The inverted "V" type of electrophoresis apparatus (Flynn and de Mayo, 1951) is used for the electrophoresis with Whatman 3MM filter paper and the same barbitone buffer used to dissolve the porphyrins. The porphyrin solution is applied to the middle of the dry Whatman 3MM filter paper in the same way as the blood serum is applied and the paper is fixed in the electrophoresis chamber with the two ends dipping in the buffer solution compartments. The buffer solution is allowed to soak up the filter paper from both ends so as to meet in the middle where the porphyrin solution has been applied.

The power supply is switched on when the whole paper is soaked with the buffer solution. About three to four hours' run under the same conditions as are used for the electrophoresis of serum proteins gives about 9 to 10 cm. separation of the porphyrins. After being dried in a hot air oven at about 60 to 70° C., the filter paper is examined in ultra-violet light and photographed to obtain a permanent record. A Hanovia-Muir table model ultra-violet lamp fitted with a 500-watt straight arc tube and

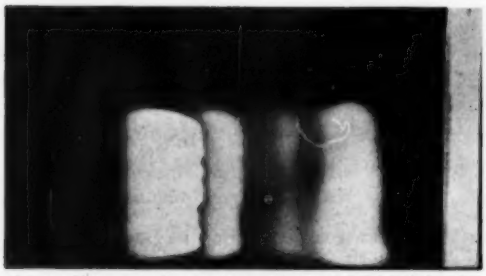

FIG. 1.—Urinary porphyrins photographed in ultra-violet light after paper electrophoresis from a case of porphyria.

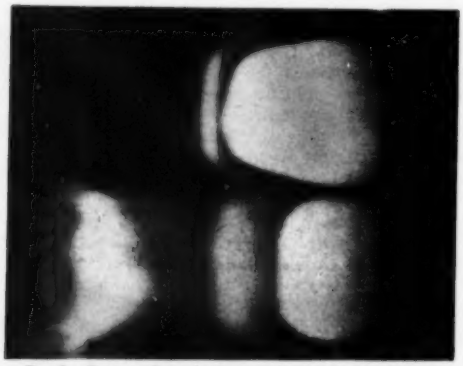

FIG. 2.—Pattern of porphyrin excretion in urine in two cases.

fluorescence filter giving ultra-violet light around 3,200 A.U. at 40 in. distance is used as the source. The separated porphyrin fractions show beautiful red fluorescence, and the area and the intensity of the fluorescence give an approximate relative proportion of the individual porphyrin fractions excreted in the urine (Figs. 1 and 2). For identification, purified porphyrins can be spotted as markers and run parallel with the porphyrin mixture extracted from the urine.

A characteristic electrophoretic pattern of porphyrin excretion can be obtained by this method and will be useful in comparing the porphyrin excretion in the same individual periodically, and also for studying the pattern of porphyrin excretion in cases of porphyrinuria and porphyria.

I am grateful to Dr. A. R. Natarajan, Chemical Examiner to the Government, and Mr. R. Viraraghavan for helping me to study and photograph the porphyrins in ultra-violet rays.

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A Container for Handling and Centrifuging Pathological Material

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(RECEIVED FOR PUBLICATION MAY 17, 1957)

For many years workers in pathology laboratories have used the "universal" container for the collection and handling of pathological material.

Although the "universal" container has proved to be a most useful bottle, it has the following serious drawbacks:

(1) Whitwell, Taylor, and Oliver (1957) found that when infected fluid in a "universal" container is spun in an angle centrifuge some of the material is sprayed over a wide area around the centrifuge, the size of the area being about 7 ft. in diameter.

If the containers are spun in a "swing-out" type of centrifuge the buckets and the outsides of the containers may be heavily contaminated, though neither the inside of the centrifuge casing nor the atmosphere is contaminated.

(2) Should at any time the cap be faulty or not properly tightened, material may escape and contaminate the outside of the bottle during normal handling.

(3) When material with a low cell content is centrifuged, cells are deposited across the whole of the floor of the bottle, and are then very difficult to recover.

In an attempt to overcome these faults, a bottle has been designed with a trough around the neck and an inner bottom surface which is conical (Fig. 1).

The bottle is made of transparent "bottle" glass, with a diameter of base 1¼ in. and overall height of 3½ in. The neck is threaded to take the standard 1 oz. bottle metal cap with rubber liner, so as to allow the rim of the cap to come below the edge of the trough wall. The depth of the trough around the

FIG. 1.—Narrow-neck container.

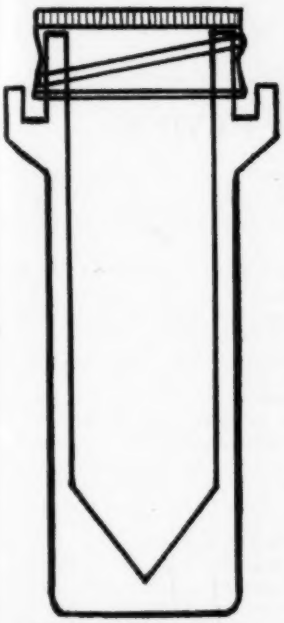

FIG. 2.—Wide-neck container.

neck is 3/16 in., and the angle of floor from periphery to centre 45°.

The design may be modified so that the container has a wider neck and is threaded so that it will take a "universal" metal screw cap and liner (Fig. 2).

Owing to the difficulty in having a single container made in glass, it was decided to employ perspex for the prototype bottle (Fig. 3). A 1 oz. narrow-necked bottle was also made in perspex to use as a control in the tests.

FIG. 3.—Perspex prototype bottle.

Tests

The perspex control bottle and a glass narrow-necked 1 oz. bottle were partly filled with 10% hydrochloric acid, tightly capped with screw caps fitted with rubber liners, and shaken. The caps were then unscrewed and immediately replaced and screwed down tightly.

The bottles were then wrapped in filter paper which had previously been soaked in Andrade's indicator and allowed to dry.

Next, the bottles were placed in a "swing-out" type of centrifuge and spun at 3,000 r.p.m. for three minutes. On removal from the centrifuge, the filter paper around both bottles showed marked staining, indicating that a considerable amount of fluid had escaped during centrifuging.

The perspex prototype container was then treated in the same way. On removal from the centrifuge the filter paper was found to be unmarked, although examination of the trough showed two large drops of fluid lying on its floor. This indicated that during the centrifuging hydrochloric acid had travelled down between the screw cap on the outer surface of the neck of the bottle and had been retained in the trough, so preventing the handling surfaces of the bottle and the inside of the centrifuge bucket from becoming contaminated by the contents of the bottle.

The bottles were then carefully washed and dried and filled with 10% hydrochloric acid using a pipette, care being taken to ensure that no fluid was allowed to come in contact with the external surfaces of the necks of the bottles. About one third of the fluid was then poured out of the bottles and they were capped tightly with dry screw caps fitted with dry rubber liners.

The bottles were then wrapped in the filter paper impregnated with Andrade's indicator and centrifuged at 3,000 r.p.m. for three minutes in a "swing-out" type of centrifuge. On examination it was found that the filter paper surrounding the 1 oz. bottles was stained, indicating that hydrochloric acid had escaped. Examination of the paper surrounding the prototype container showed it to be unmarked, although a drop of fluid lying in the base of the trough could be seen.

The above tests were carried out several times in the "swing-out" type of centrifuge, and also in an angle centrifuge, always with a similar result.

A suspension of approximately 1,000 red blood cells per c.mm. was made in physiological saline, and 10 ml. of this fluid was placed in the prototype container, and also in a 1 oz. bottle. Both containers were centrifuged at 3,000 r.p.m. for three minutes and the red cell deposits examined. It was found that in the container with the conical bottom there was a compact "button" of cells which remained after the supernatant fluid had been carefully decanted. In the 1 oz. bottle the red cells were found to be distributed across the floor of the bottle with the majority of cells lying around the periphery of the floor, this being due to the slight convexity of the floor of the container. After carefully decanting the supernatant

fluid the remaining cells were found to be intermixed with the residual supernatant fluid, and were very difficult to recover in a useful concentration.

Summary

A container has been described for the centrifuging and handling of pathological material.

The advantages of the container described are:

(1) Prevention of widespread contamination when centrifuging; (2) prevention of contamination of the outside of the container when pouring; (3) prevention of contamination of the outside of the bottle by the fitting of a loose or faulty cap; (4) enabling a "button" to be formed when centrifuging in a "swing-out" type of centrifuge, thus saving the cost of conical centrifuge tubes.

We wish to thank Minerva (Cardiff), Ltd., for making the perspex containers.

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BOOK REVIEW

Disorders of the Blood, 8th ed. Sir Lionel Whitby and C. J. C. Britton. (Pp. viii+878; 20 colour plates and 125 figs. £3 15s.). London: Churchill. 1957.

The eighth edition of "Whitby and Britton" finally appeared after the death of the senior author, and, although the galley proofs had been almost completed before he died, the final preparation of the eighth edition must have been an additional burden at an unfortunate time for Dr. Britton. The fact that there have been eight editions in 22 years not only shows that the popularity of the best-known English textbook of haematology is being maintained, but is also clear proof of the enormous growth of the subject during this time. Since the last edition in 1953 so much new work has appeared—and in fact so many new journals of haematology and allied subjects—that the authors have had to add considerably to the subject-matter. This has been done effectively, and no fault can be found with the up-to-date information available, but the authors rightly point out that the book is now no longer a simple textbook on haematology but is really now a reference book for haematologists and haematological laboratories.

The authors, however, have not been quite so successful with the elimination of the old and now obsolete material, and this needs ruthless pruning in future issues, because it leads to disconnected chapters beginning with introductory material bearing no relationship to modern views and more recent work. The photomicrographs are also of variable standards, and many of the older ones could be omitted without loss, e.g., Fig. 39, p. 240 (which is no less than shocking), shows a macropolycyte which is much better seen in the colour Plate XI two pages previously. Fig. 10, which purports to be leucoblastic hyperplasia in a bone marrow section, is also quite meaningless. Some of the new black-and-white photomicrographs, for example, Figs. 71 and 72 and Plate XIV, do demonstrate what can be achieved by enlarged black-and-white photographs aiming to show detail.

The book shows every sign of remaining the leader in its field and maintaining its well-deserved popularity. The references are more than adequate, and the price of 75s. compares most favourably with other publications of this size.

A. GORDON SIGNY.

J. clin. Path. (1958), **11**, 289.

A RE-ASSESSMENT OF THE GASTRIC LESION IN PERNICIOUS ANAEMIA

BY

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(RECEIVED FOR PUBLICATION JANUARY 9, 1958)

This short review is based on the Kettle Memorial Lecture for 1957. In order to understand the localization of the lesion found in the stomach in pernicious anaemia and the histological changes that occur in the affected mucosa it is necessary to remind ourselves of the normal anatomy and histology. Fig. 1 shows the average normal distribution of the body and pyloric mucosae in the human stomach. The body mucosa area occupies about the upper two-thirds of the stomach and contains the gastric glands proper. The pyloric mucosa area occupies about the lower third of the stomach and contains the pyloric glands. These mucosal areas are not sharply defined from each other and at the borderline the glands of one mix with the glands of the other. Along the lesser curvature the body glands usually meet the pyloric glands somewhere in the region of the incisura angularis. Along the greater curvature the body glands extend further into the pyloric region and may almost reach the pylorus. The pyloric region, therefore, is commonly triangular in shape with its apex at the incisura angularis. However, the distribution of the pyloric mucosa along the lesser curvature is very variable and, on occasion, it may extend right up to the oesophagus as a narrow band. This observation may well be of importance in the understanding of the topography of chronic ulcers in the stomach.

The surface epithelium of the body and pyloric mucosae has everywhere the same structure. It is composed of tall, regular columnar cells with basal nuclei and clear, transparent, only faintly granular cytoplasm. Cells with striated borders and goblet cells are not found amongst the cells of the surface epithelium in normal stomachs and only occur when islets of intestinal epithelium are present (Magnus, 1937).

The body mucosa (Fig. 2) contains the gastric glands, simple branched tubules opening into the gastric pits which, in the body mucosa, are shallow. The tubules are tightly packed together and arranged perpendicular to the surface of the mucosa. The blind ends are club-shaped and sometimes divide into two or three branches. These glands contain four types of cell: (1) Neck chief cells, (2) body chief cells, (3) parietal cells, and (4) argentaffine cells.

The neck chief cells are identical with the cells lining the cardiac and pyloric glands. They are

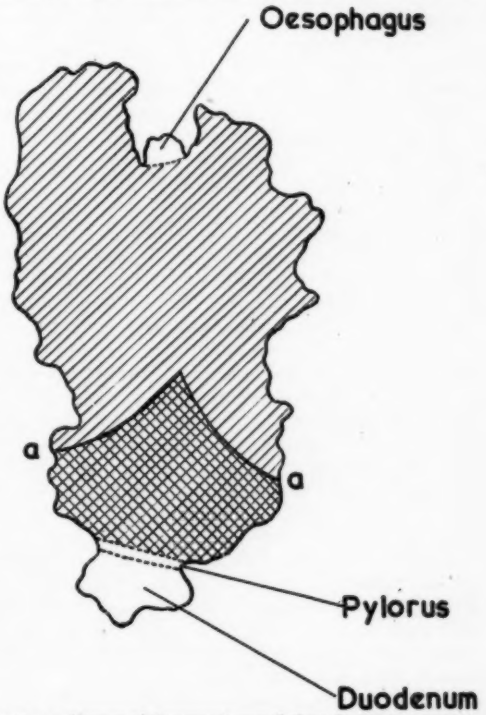

FIG. 1.—Diagram of the stomach opened along the greater curvature. Line a—a represents the demarcation between body and pyloric mucosae.

mucin-producing columnar cells which line the upper, narrower part of the glands. The body chief cells produce the pepsin of the gastric juice which is present in the cells in the form of a granular precursor. They line the lumina of the tubules below the neck chief cells. The parietal cells produce the acid of the gastric juice and are scattered singly, internal to the body chief cells, but are most numerous at the necks of the tubules where they are interspersed between the neck chief cells. They are spherical or triangular in shape and the cell cytoplasm stains readily with acid dyes. Very occasionally an argentaffine cell is present at the bases of the tubules.

The pyloric mucosa (Fig. 3) is also composed of glands with simple, branched tubules, but the tubules are coiled and the glands open into gastric pits which extend much deeper inwards and are frequently branched. The pyloric glands are lined by mucin-producing cells only. Here, also, an occasional argentaffine cell may be found.

In both the body and pyloric mucosae the glands are separated by scanty connective tissue consisting of a delicate network of collagenous and reticulum fibres in which cells are sparse. These consist of occasional fibroblasts and reticuloendothelial cells and scattered lymphocytes, plasma cells, and eosinophils. In normal mucosa lymphocytes may occasionally form small lymph follicles, especially in the pyloric mucosa.

Before considering the changes that may be found in the gastric mucosa in anaemia it would be as well to review briefly the history of the association of a lesion of the stomach with the clinical condition we call pernicious anaemia. This extends over nearly 100 years, for it was Austin Flint (1860) who first suggested the possibility of such a relationship. Samuel Fenwick (1880) made the first pathological investigation of the stomach in pernicious anaemia. He recorded four cases of "essential anaemia," in all of which there was atrophy of the stomach and especially of the gastric mucosa. Nothnagel (1879), almost at the same time, described similar findings, but in all these cases the stomachs were unfixed and the histology uncertain. Faber and Bloch (1900, 1904) published the first reliable histological description of changes in the stomach based on well-fixed material. They examined the stomachs from eight patients dying from pernicious anaemia and found a diffuse gastritis in every case. They made no menton of any localization of the gastritis to any particular part of the stomach. From 1900 to 1938 it was generally accepted that a "diffuse chronic gastritis" was present in the stomach in pernicious anaemia. Wallgren (1923–25) and Brown (1934) confirmed Faber and Bloch's findings but, for the most part, on unfixed material. Magnus and Ungley (1938) published the results of an investigation of the stomachs of seven cases of pernicious anaemia in which the stomachs were fixed soon after death. They showed that, in these cases, the lesion was confined to the body mucosa area of the stomach and that the pyloric mucosa area was unaffected. Meulengracht (1937), at the first meeting of the German Haematological Society held at Munster, described similar findings which were exactly the reverse of what he had expected from his work on the stomach of the pig. Cox (1943) confirmed these results and Magnus (1952) recorded the same findings in a further 10 cases.

It would be as well to consider here the nature of the lesion which these workers described and the tentative conclusions which could be made, at that time, from their observations. The macroscopic changes seen in the stomach were usually striking. The upper two-thirds of the stomach, that is, the region of the body mucosa, showed a severe atrophy so that this area was reduced to the thinness of parchment. At the junction of the body with the pyloric mucosa, however, there was a transition, usually abrupt, to the normal thickness of the stomach wall and this was maintained throughout the whole of the pyloric antrum. Histological examination confirmed the naked-eye distribution of the atrophy. In the body mucosa area there was a severe atrophy involving all the coats of the stomach wall (Fig. 4) and it was clear that the parchment-like thinness observed macroscopically was not due to mucosal atrophy alone. In the mucosa (Fig. 5) all that remained was the surface epithelium and a few scattered glands lined by mucus-producing cells whilst the specialized body chief cells and parietal cells had disappeared. Yet there was a complete absence of fibrosis, cellular infiltration, obliterative endarteritis, or other evidence of past inflammation, and there was a corresponding absence of fibrosis in the submucosa and subserosa. The muscle coat showed atrophy, but here also there was an absence of fibrosis. In many stomachs examined large areas of intestinal epithelium were present in the atrophic body mucosa. The significance of this finding will be discussed later.

The main conclusions which could be drawn from this work were as follows:

(*a*) The lesion that occurred in the stomach in pernicious anaemia was not the end-result of an

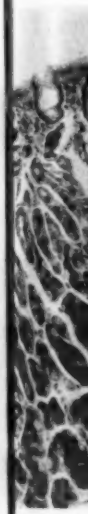

FIG. 2.–

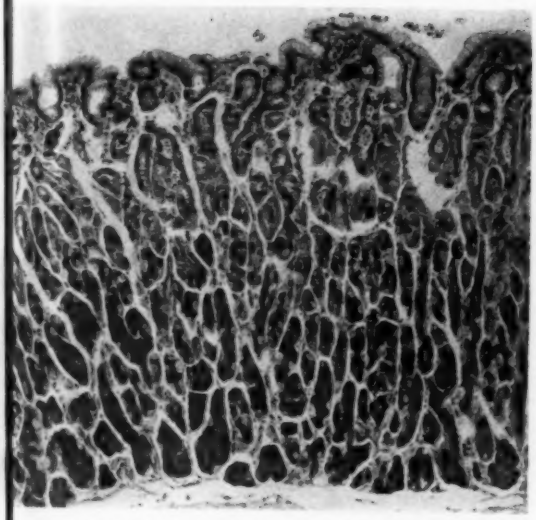

FIG. 2.—Normal body mucosa (Lillie's azure A—eosin B, × 95).

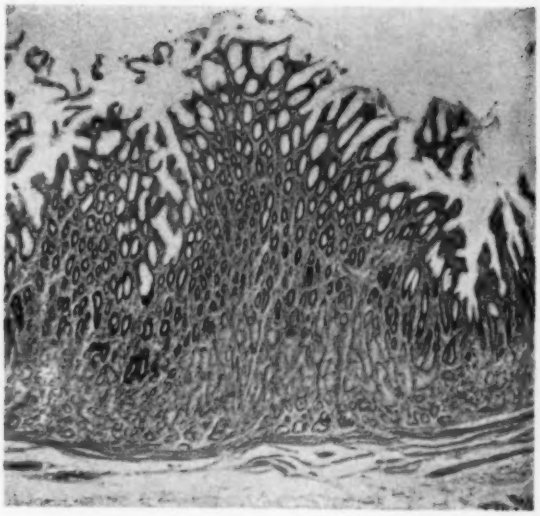

FIG. 3.—Normal pyloric mucosa (haematoxylin and eosin, × 30).

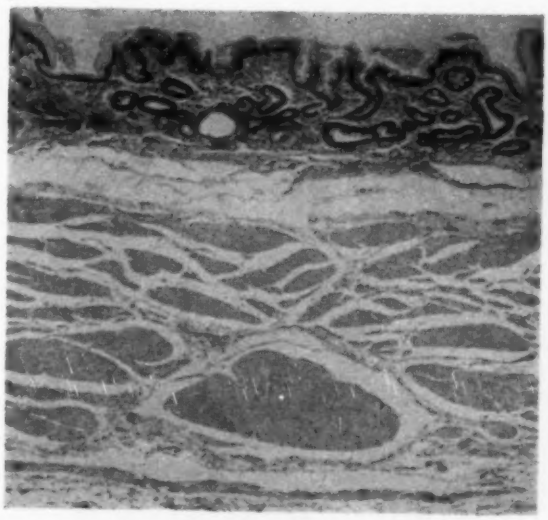

FIG. 4.—Severe atrophy involving all coats of the stomach wall (haematoxylin and eosin, × 50).

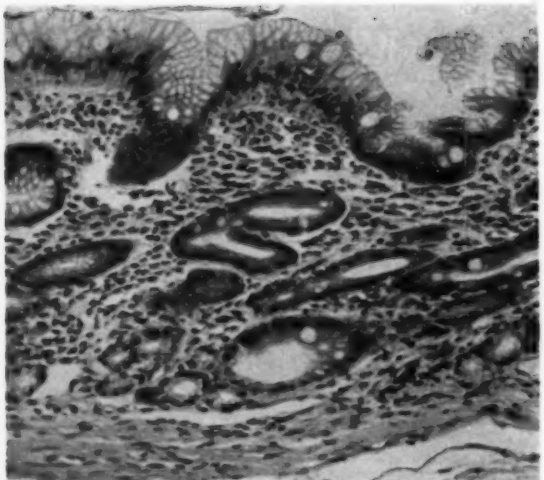

FIG. 5.—The body mucosa is completely replaced by simple glands lined by mucus-producing cells. There is no evidence of a past or recent inflammatory reaction (haematoxylin and eosin, × 200).

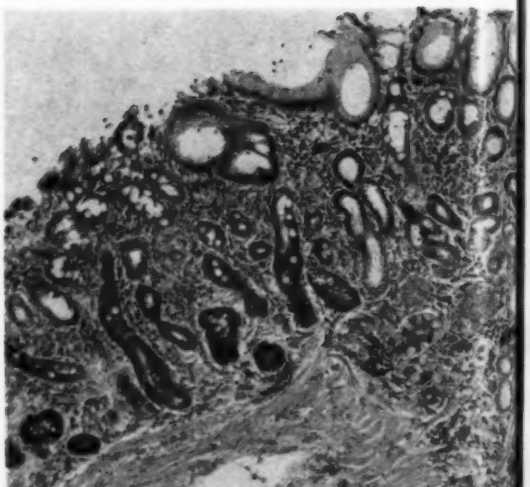

FIG. 6.—Severe atrophic gastritis with intestinal metaplasia. Note the persistence of recognizable body mucosa to the left of the picture (Lillie's azure A—eosin B, × 45).

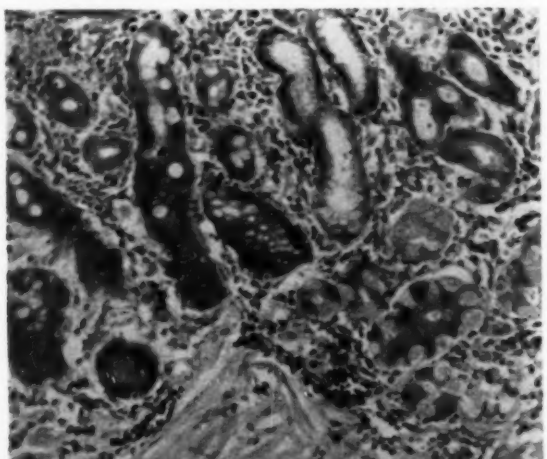

FIG. 7.—A higher power view of Fig. 6 showing undoubted body chief and parietal cells (Lillie's azure A—eosin B, × 160).

inflammatory gastritis but appeared to be the result of some endocrine or nutritional deficiency or might even be congenital in origin.

(*b*) At this time this lesion had not been seen in any other condition and most workers believed that it was specific for pernicious anaemia.

With the introduction, in 1949, of the flexible gastric biopsy tube by Wood and his colleagues at the Walter and Eliza Hall Institute of Medical Research, Melbourne, a milestone was reached in the investigation of the changes that might occur in the stomach, not only in pernicious anaemia, but also in other anaemias, notably iron-deficiency anaemia. A method was available for studying the state of the gastric mucosa in life which was not only safe but could be repeated several times on the same patient. One had no longer to wait for many years to collect a small amount of well-fixed material from dead patients but could gather, in a relatively short time, more material and information than one individual could collect previously in a lifetime. The investigation of patients with pernicious anaemia and iron-deficiency anaemia over the last eight years has produced new evidence which must now be considered and as a result of which some of our previously held views must be modified.

In 1955 Joske, Finckh, and Wood reviewed 1,000 gastric biopsies performed on 623 subjects, including 100 cases of pernicious anaemia or subacute combined degeneration of the cord or both. Their findings in these cases are shown in Table I. All these patients had histamine-fast achlorhydria. From Table I it can be seen that the gastric lesion, previously described as occurring in pernicious anaemia, was only present in 40 out of the 100 cases investigated. In 60 cases only varying degrees of what, from the description given, would appear to be inflammatory atrophic gastritis were present. There was a widespread cellular infiltrate of the substantia propria with atrophy of tubules. Survival of body chief and parietal cells varied with the severity of the lesion, but usually a few survived even in the severest form of atrophic gastritis. Within the last two years the writer has seen a stomach from a man, aged 74, who had had pernicious anaemia for seven years. This showed similar changes. Instead of the severe non-inflammatory atrophy of the body mucosa with complete disappearance of the specialized cells there was a severe atrophic gastritis with persistence of foci of body chief and parietal cells (Figs. 6 and 7).

TABLE I

RESULTS OF GASTRIC BIOPSY ON 100 CASES OF PERNICIOUS ANAEMIA OR SUBACUTE COMBINED DEGENERATION OF THE CORD OR BOTH (JOSKE, FINCKH, AND WOOD, 1955)

Number of Cases	State of Gastric Mucosa
9	Superficial gastritis with atrophy
16	Atrophic gastritis
35	Severe atrophic gastritis
40	Gastric atrophy
100	

It would seem, therefore, that the lesion in the stomach in pernicious anaemia may, in many cases, be less severe than complete gastric atrophy and in the nature of an inflammatory atrophic gastritis. Palmer (1954), in a revaluation of the pathology of gastritis based on gastric biopsy material, goes so far as to state that he found no evidence that a distinction could be made between "chronic atrophic gastritis" and "mucosal atrophy" in the stomach in pernicious anaemia or that glandular atrophy may develop without chronic inflammation.

Most observers would feel to-day, however, that in adult pernicious anaemia two types of gastric lesion may be found, one an atrophic gastritis of varying severity and the other a gastric atrophy involving all coats of the stomach wall. What relationship, if any, do these two lesions have to each other? Witts (1956) believes that they are separate entities with possibly different aetiologies. Whilst this may be so, it seems to the writer, having re-examined his own material and had the opportunity of examining other workers' material obtained by gastric biopsy, that they represent stages in the same pathological process. Such a conception has already been put forward by Davidson and Markson (1955).

How is it that, in the post-mortem material examined before the introduction of the flexible gastric biopsy tube, only gastric atrophy in its severest form was seen and involving all coats of the stomach wall? There seem to be two possible explanations. First each observer only saw a relatively small number of cases, and secondly all the patients were dead either from untreated pernicious anaemia or from some other disease, having had pernicious anaemia for many years. That the gastric lesion in pernicious anaemia is the end-result of some inflammatory or degenerative process would also explain why it has never been seen in childhood: it cannot be congenital in origin. The possibility that the lesion is the result of an inflammatory process does not necessarily mean that its cause must be exogenous. Recently papers have appeared reporting the results of investigations of the stomach in iron-deficiency anaemia which may well throw light on

the aetiology of the gastric lesion in pernicious anaemia. Davidson and Markson (1955) and Badenoch, Evans, and Richards (1957) have investigated the state of the gastric mucosa in iron-deficiency anaemia by gastric biopsy. In the series of cases in both papers a very high percentage of patients showed an abnormal gastric mucosa. Many had severe chronic atrophic gastritis and some complete gastric atrophy. Both sets of authors produce cogent arguments and evidence to support the view that the gastric changes are the result of the iron deficiency and not the cause of it. This conception opens up a new approach to the problem of the part played by the stomach in the pathogenesis of pernicious anaemia. The views put forward by these workers, however, have been challenged in a recently published paper by Lees and Rosenthal (1958), who investigated 21 patients with iron-deficiency anaemia. These patients all had gastric biopsies, their blood was restored to normal by iron therapy, and in 19 cases gastric biopsy was repeated in a year. In no case was there any improvement in the state of the gastric mucosa, and in some the changes had progressed. It was concluded, therefore, that the gastric mucosal lesions were not due to iron deficiency but preceded and favoured its development.

There can be no doubt that gastric atrophy of the severest degree can occur without the clinical picture of pernicious anaemia. As has been seen it occurs in iron-deficiency anaemia and also, as in Joske *et al.*'s (1955) series, without any anaemia at all. Doig, Motteram, Weiden, and Wood (1950) describe its presence in a perfectly healthy medical student who had histamine-fast achlorhydria. Of special interest is the fact that his maternal grandmother had pernicious anaemia. Why do these patients not have the clinical picture of pernicious anaemia ? It is known that if the whole stomach is removed in man it takes from four to six years for pernicious anaemia to develop. Badenoch *et al.* (1957) showed in a series of cases of iron-deficiency anaemia that some patients had impaired absorption of labelled B_{12}, and in four out of six patients who were given 50 mg. of intrinsic factor with labelled B_{12} there was a significant increase of absorption of the vitamin. Pedersen, Lund, Ohlsen, and Kristensen (1957) estimated the incidence of mild vitamin B_{12} deficiency in elderly patients with histamine-refractory achlorhydria. Forty-six patients aged 50 or more had several studies of sternal marrow and seven were shown to have partial or incipient megaloblastic erythropoiesis. Unfortunately no gastric biopsies were performed. Mollin, Booth, and Baker (1957) record an investigation into the absorption of labelled vitamin B_{12} in control subjects, in Addisonian pernicious anaemia, and in the malabsorption syndrome. They found five patients with histamine-fast achlorhydria who did not have pernicious anaemia yet gastric biopsy showed gastric atrophy or atrophic gastritis. These patients absorbed as little labelled vitamin B_{12} as did patients with pernicious anaemia, but they had no clinical or other pathological evidence of this disease. All this surely points to the hypothesis that, if the manufacture of the intrinsic factor or its precursors is gradually reduced, the development of the gastric lesion and a subclinical deficiency of vitamin B_{12} run hand in hand.

That severe gastric atrophy may be present for years before clinical pernicious anaemia develops is shown by the case recorded by Robertson, Wood, and Joske (1955). This was a man aged 65 who had a long history of dyspepsia. Gastric biopsies showed complete gastric atrophy yet his peripheral blood and bone marrow were normal. Five years later he returned with typical pernicious anaemia and subacute combined degeneration of the cord.

There is ample pathological evidence to show that after adequate therapy the gastric mucosa in pernicious anaemia shows no recovery. This is what one would expect if the cause of the lesion is an absence of intrinsic factor or its precursor. In the writer's material four patients had been adequately treated for from five to 13 years and all showed severe gastric atrophy. Doig and Wood (1950), Finckh and Wood (1953), Joske *et al.* (1955), Badenoch *et al.* (1957), and others have all produced overwhelming evidence that the gastric mucosa does not recover after prolonged therapy.

A fascinating aspect of the problem is revealed by the study of the literature on juvenile pernicious anaemia. Many workers dismiss out of hand the fact that true pernicious anaemia can occur in childhood or adolescence, believing that all these cases can be explained by the association of a megaloblastic anaemia with sprue or a similar syndrome. This is not the case, and after the application of the strictest criteria one is left with about 20 cases of true juvenile pernicious anaemia. One of the most interesting points about these cases is that many have free HCl in the gastric juice, a fact that would make many refuse to accept them as pernicious anaemia out of hand, but this would be unwise. Mollin, Baker, and Doniach (1955) record the case of a young man, aged 18

years at the time of the report, who had suffered from pernicious anaemia from the age of 13 months. His parents were first cousins. The patient secreted normal amounts of acid and pepsin in the gastric juice which, however, was deficient in intrinsic factor. A gastric biopsy showed perfectly normal body mucosa. The patient's father also suffered from pernicious anaemia with achylia gastrica and a gastric biopsy showed a severe atrophic gastritis with some parietal and body chief cells still recognizable, i.e., gastric atrophy was not yet complete. The study of these rare cases of true juvenile pernicious anaemia indicates, as Benjamin (1948) has pointed out, that they may be explained by a congenital absence of intrinsic factor or its precursor. The presence in some of free HCl with a normal mucosa suggests that it may take many years for the gastric lesion to develop, and supports the conception that a subclinical state of vitamin B_{12} deficiency may be present for a long time before the clinical picture of pernicious anaemia is seen.

The fact that there is often a strong family history in many cases of pernicious anaemia has been known for many years. One of the best investigations into the subject has been carried out by Mosbech (1953) in Copenhagen. He investigated approximately 3,000 relatives of 234 patients with pernicious anaemia and found that the disease occurred about 20 times more frequently amongst them than in a control series. Its distribution amongst various categories of relatives appeared to be compatible with recessive inheritance with a reduced rate of manifestation. McIntyre, Sachs, Krevans, and Conley (1956) have shown that the family members of patients with pernicious anaemia not infrequently show subnormal absorption of radioactive B_{12}. Surely these results also point to an inborn chemical error as the basic cause of both pernicious anaemia and the gastric lesion with which it is associated.

It is generally agreed, but not proved, that it is the neck chief cells near the surface of the body mucosa of the stomach which secrete the intrinsic factor, about the chemical nature of which little is known. Marmion, Gardner, Saint, and Stubbe (1953) agree that there is good evidence that the intrinsic factor is a mucoprotein, but not one that is reduced by a receptor-destroying enzyme, and it differs therefore in its properties from a gonadotrophin and mucoprotein derived from respiratory tract cells. The complexity of the work is well seen in a paper by Glass, Stephanson, Rich, and Laughton (1957), who investigated intrinsic factor activity of human gastric juice after fractionation by continuous electrophoresis on paper curtains. Intrinsic factor activity was so widespread throughout the fractions that they suggest that intrinsic factor is, perhaps, not a substance but only a reactive group, present on one or more mucoproteins endowed with intrinsic factor activity.

For the last 20 years it has been suggested from time to time that there is an increased incidence of gastric cancer in pernicious anaemia. There are two ways of considering figures in relation to this problem. First the number of people of both sexes who have pernicious anaemia and develop gastric cancer may be studied. Table II shows

TABLE II
INCIDENCE OF GASTRIC CANCER IN PATIENTS WITH PERNICIOUS ANAEMIA

Source	No. of Patients (Both Sexes)	Period of Time (years)	No. of Cases of Gastric Cancer	Approximate No. of Cases per 1,000 Patient-Years
Wilkinson (1956)	2,211	28	39	1·0–2·0
Davidson (1957)	726	10	7	1·9

the figures for the incidence of gastric cancer in two large pernicious anaemia clinics in this country. As large numbers of patients are being followed up for varying periods of time the last column in the table shows the approximate number of cases per 1,000 patient-years which, under these circumstances, is the most convenient way of measuring the rate of occurrence of a disease. Whilst there are no reliable comparable figures for the frequency of cases of gastric cancer in the population as a whole, one can make a reasonable guess in the following way. In 1955 there were 7,000 deaths in England and Wales from cancer of the stomach in both sexes between the ages of 40 and 70. There must be perhaps between 8,000 and 10,000 new cases per year to account for this number of deaths. The population of the country in this age group is approximately 16,000,000 and, if incidence is defined as meaning new cases per 1,000 patient-years, then the figure for cancer of the stomach is 0.5 to 0.6. It would appear, therefore, that the chance of acquiring cancer of the stomach may be two or three times as great in a patient with pernicious anaemia as in the population as a whole.

Secondly the mortality from gastric cancer in patients with pernicious anaemia can be considered and can be compared with the mortality from gastric cancer in a population of the same age group. Table III shows figures from three different sources, and the proportion of deaths due to gastric cancer are in close agreement, showing

TABLE III
MORTALITY DUE TO GASTRIC CANCER IN PATIENTS WITH PERNICIOUS ANAEMIA

Source	No. of Patients Dying with Pernicious Anaemia (Both Sexes)	Percentage of Deaths Due to Gastric Cancer
Kaplan and Rigler (1945)	293	12·3
Wilkinson (1949)	301	9·3
Cappell (1957)	54	12·9

an average of 11.5%. In 1955, 3.7% of all deaths in England and Wales in people of both sexes between the ages of 40 and 70 were due to gastric cancer. There would seem to be agreement, therefore, between the figures obtained in two different ways from which it may be concluded that the chances of a patient with pernicious anaemia developing cancer of the stomach are about three times greater than might be expected in the population as a whole. The situation has been well summed up by Mosbech and Videbaek (1950), who, as a result of a follow-up of 301 patients being treated for pernicious anaemia over a 20-year period, concluded that there was no increased mortality in this disease compared with the population as a whole. Although the total death rate from cancer in all organs did not appear to be excessive, the number of patients with gastric cancer greatly exceeded the expected figure and there was the same increase for both sexes—the lónger you live with pernicious anaemia the more likely are you to develop gastric cancer.

Why should patients dying with pernicious anaemia have this increased incidence of gastric cancer which, according to Mosbech (1953), also occurs amongst their relatives without pernicious anaemia? The usually accepted explanation is that the two diseases may have an inherited genetic background. This may be so, but it is possible that there is another explanation. In the writer's material it has been extremely common to find that most of the body area of the stomach, the site of the gastric lesion, has been replaced by intestinal epithelium and this observation has been confirmed by others. It is, in fact, most unusual to see such extensive intestinal metaplasia in the body area of the stomach except in pernicious anaemia. Morson (1955a, b, and c) in several papers has published the results of a painstaking investigation into the incidence and distribution of intestinal epithelium in the stomach in simple ulcer, in cancer, and in duodenal ulcer. He has shown that about 30% of gastric cancers arise from areas of intestinal metaplasia which should be regarded, therefore, as a precancerous state. The natural deduction from this work is that cancer of the stomach should be much commoner in the fundic area in cases of pernicious anaemia than when it arises without this association. According to Schell, Dockerty, and Comfort (1954), this is indeed the case. They investigated 94 cases of pernicious anaemia with gastric cancer and found that in 58.3% it was in the fundic or cardiac regions and in 14.6% there were either separate tumours in the pyloric and fundic areas or an extensive tumour involved both areas. These figures have so far not been confirmed by other observers, some of whom, indeed, have published figures the exact opposite, but the largest number of patients under observation was 14, so that there can be no comparison. Reports from other clinics on this important question of the siting of gastric cancer in cases of pernicious anaemia must be awaited.

To summarize, what is the position to-day with regard to the gastric lesion in pernicious anaemia? Twenty years ago it was believed to be a specific lesion and not the end-result of an atrophic gastritis. It would now seem that both these conclusions have to be modified and the new evidence suggests that the basic lesion in pernicious anaemia does not lie in the stomach. The changes in the gastric mucosa may well be endogenous in origin and due to the fact that the intrinsic factor or its precursors are necessary for the maintenance of its normal structure and function. The basic lesion would appear to be an inherited chemical defect, perhaps expressed by the inability of certain cells of the gastric mucosa to manufacture an essential enzyme.

My thanks are due to Sir Stanley Davidson for kindly allowing me to use unpublished figures in Table II and to Professor D. F. Cappell for unpublished figures in Table III. I am indebted to Mr. M. P. Curwen, medical statistician to St. Bartholomew's Hospital and King's College Hospital Medical School, for advice on the figures for the incidence of gastric cancer in pernicious anaemia. Mr. George Harwood is responsible for the photomicrographs.

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RENAL PAPILLARY NECROSIS IN CHILDHOOD

BY

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Necrosis of the renal papillae is an uncommon lesion in adults, and, judging from the scarcity of reports in the literature, must be even less common in childhood. In adults it is usually associated with diabetes mellitus, suppurative pyelonephritis, and chronic urinary obstruction. The course of the disease is generally acute, and in the presence of infection clinically difficult to distinguish from suppurative pyelonephritis. The lesion may be bilateral or unilateral and may involve only the tip of the papilla or the entire pyramid up to the cortico-medullary junction. It is frequently overlooked at necropsy.

So far only three reports of the lesion occurring in childhood have been traced. Edmondson, Martin, and Evans (1947) reported it in a child of 11 years with a urinary infection, and Tamaki and Whitman (1952) in a 2-month-old infant with a gastro-intestinal disorder. Swartz (1954) described the condition in an infant aged 5 months with meningitis, and in this instance the lesion was associated with thrombosis of the renal interlobar veins.

The main purpose of this paper is to report four cases in children which have come to necropsy at this hospital since 1950, and to suggest a pathogenesis for one of them.

Case Reports

Case 1.—A child aged 4 years and 9 months was transferred to the Royal Victoria Infirmary on November 8, 1951, eight days after an accident in which she sustained extensive burns involving the whole trunk, left buttock, and both arms. Haemoglobin was 66% and she was transfused with blood. On the twelfth day after admission her condition was poor and she was given oxygen, and chloromycetin was added to the penicillin which had previously been given. A further transfusion was given, but her condition deteriorated and she died 22 days after the accident.

Laboratory Findings.—On November 20 analysis of urine showed 4 R.B.C.s and 240 pus cells per c.mm., a trace of albumin, and 2.5 g./litre chlorides. On the next day 320 mg.% sodium, 397 mg.% chlorides, and 25.1 mg.% potassium. The alkali reserve was 32.8 ml. CO_2/100 ml. serum. The concentration of urea in the cerebrospinal fluid was 80 mg.%.

Ps. pyocyanea was detected in blood culture and was sensitive to chloromycetin.

Necropsy Findings.—Both kidneys were swollen and showed on the capsular and cut surfaces scattered groups of small abscesses in the cortices and medullae. The medullae were deeply congested and the cortices relatively pale whilst on the right side there was an irregular zone of anaemic infarction extending to the capsular surface. The bladder was inflamed. The ureters were normal. The renal veins were patent.

Of the remaining organs, the heart was normal, the liver congested, and the lungs the seat of a bronchopneumonia.

The kidneys showed on each side a suppurative pyelonephritis with colonies of pleomorphic Gram-negative bacilli scattered among the inflammatory exudate. The papillae of the left kidney were necrotic, but dilated collecting tubules stuffed with debris and degenerate leucocytes could be distinguished. Zones of inflammation separated the necrotic tissue from the viable renal parenchyma. Convoluted tubules were dilated and contained eosinophilic material, but the glomeruli were unaffected except for those involved by the suppuration and a small recent infarct on the right side. Small venous thrombi were seen on both sides in the vicinity of the suppuration and a few tubulo-venous aneurysms were noted in the juxtamedullary areas.

The liver showed a well-marked centrilobular necrosis.

The pancreas appeared normal.

The right adrenal showed small haemorrhages and thrombi with limited cortical necrosis, and in the left the medulla was infiltrated by a ganglioneuroma.

This was a case of unilateral papillary necrosis in a child suffering from burns, pyocyanea septicaemia, and acute suppurative pyelonephritis.

Case 2.—This baby girl aged 8 weeks was admitted on April 10, 1950, with a three-week history of passing green stools. On examination she was an ill, dehydrated baby with palpable kidneys, and the urine contained albumin, red blood corpuscles, and pus cells. She was treated with penicillin and sulphonamides. Four days after admission, despite the absence of vomiting and diarrhoea, she was de-

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FIG. 1.—Case 1: a necrotic papilla and abscesses in the medulla and cortex. Haematoxylin and eosin, × 4.

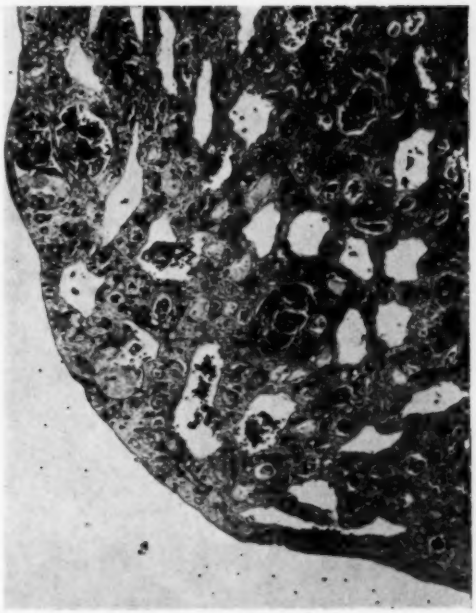

FIG. 2.—Case 1: dilated necrotic tubules filled with casts. Haematoxylin and eosin, × 180.

hydrated, with a blood urea of 187 mg.%, and fluids were given by subcutaneous drip. Ten days after admission she suddenly became worse and in a short time the fontanelle had become grossly sunken. Eschatin was given four hourly. Five days later the blood sodium had risen from 270 mg. to 430 mg. and the eschatin, together with normal saline which was being given by mouth, was stopped. Thereafter the child's condition worsened and 20 days after admission the breathing became periodic, the skin cold and mottled, and she died.

Laboratory Findings.—Analyses of urine gave the results per c.mm. set out below:

Date	Red Blood Cells	Pus Cells	Casts
14/4/50	Few	Occasional	0
15/4/50	30	91	3
19/4/50	4	12	
26/4/50	Few	1,200	
28/4/50	—	490	

Albumin + in all specimens and heavy growth of coliform organisms. Chlorides, 2·85–5 g./litre.

Blood chemistry gave the following results:

	Urea (mg.%)	Sodium (mg.%)	Chlorides (mg.%)	Potassium (mg.%)
14/4/50	187	—	—	—
17/4/50	120	270	288	28·6
	Alkali reserve 46 vol. CO_2 per 100 ml. serum			
21/4/50	76	268	—	21·9
25/4/50	148	433	—	—

Necropsy Findings.—Both kidneys were swollen with pale cortices and showed numerous small abscesses, but the most striking lesion was a necrosis of all the papillae which were a pale yellow. The pelves were congested and one side contained purulent material. The ureters were congested and one contained purulent material. The bladder was not obviously inflamed but showed a few petechial haemorrhages in its wall.

Except for some dilatation of the heart and oedema of the lungs the remaining organs were normal.

The necrosed papillae of the kidneys were separated from viable tissue by a zone of inflammatory reaction. Dilated collecting tubules containing amorphous material and Gram-negative bacilli could just be distinguished in areas of coagulation necrosis. All of the papillae examined were affected in varying degree and some separating from the living tissue showed obvious fibroblastic activity at their bases. Numerous calcified casts were seen in the second convoluted tubules, many of which were dilated, lined by atrophic epithelium, and contained eosinophilic material together with a few polymorphonuclear leucocytes. The glomeruli, except for those involved by suppuration, appeared normal and no thrombi or significant change was seen in the blood vessels.

The pancreas was normal.

This was a case of bilateral papillary necrosis complicating acute suppurative pyelonephritis.

FIG. 3.—Case 2: a necrotic papilla separating from its pyramid. Haematoxylin and eosin, × 6.

FIG. 4.—Case 2: the line of sequestration at a higher magnification. Haematoxylin and eosin, × 150.

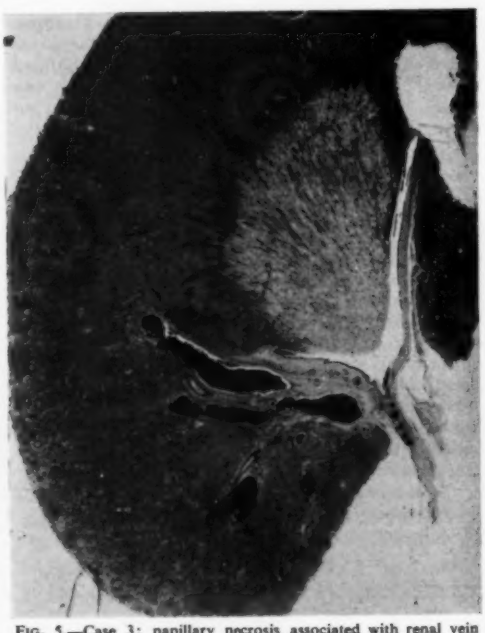

FIG. 5.—Case 3: papillary necrosis associated with renal vein thrombosis. Haematoxylin and eosin, × 6.

FIG. 6.—Case 3: zones of hyperaemia and reaction at the base of the necrotic papilla. Haematoxylin and eosin, × 180.

The role of other factors can only be a matter of conjecture, but "eschatin" had been given and there was a severe lower nephron lesion.

Case 3.—A baby boy aged 7 months was admitted on December 30, 1951, with a history of cough from the age of 3 months. Three days before admission he began to breathe heavily, became listless, and was feverish. These symptoms gradually worsened and on the day of admission he had a fit. On examination he was an ill baby with a mottled cyanosis all over the trunk and extremities. His pulse was impalpable but hydration was moderately good. At no time while in hospital did the baby appear to be taking any notice of his surroundings, and five days after admission it was felt that he had severe irreparable cerebral damage as shown by his fluctuating temperature, occasional irregular respirations, and failure to respond to his surroundings. On the day of his death, nine days after admission, his urine contained albumin and 5 pus cells per c.mm.

Necropsy Findings.—The apices of the pyramids of the kidneys showed irregular areas of discoloration and yellowish streaking. In each renal vein a grey thrombus was seen beginning within one centimetre of the inferior vena cava and extending into the kidney substance. The ureters were patent. The mucosa of the bladder was healthy.

The brain appeared normal.

The heart showed some dilatation of its ventricles and the lungs a purulent bronchitis and bronchopneumonia, but the remaining organs were normal.

The papillae of the kidneys were involved by a coagulation necrosis in which only ghost forms of dilated cast-filled collecting tubules could be discerned. These necrosed papillae were separated from viable tissue by young vascular connective tissue. The pelvic and arcuate veins were occluded by organized thrombi whilst the interlobular veins and those of the perinephric tissue were patent and dilated.

The glomeruli appeared healthy, but many lower nephrons, particularly the second convoluted tubules, were dilated and contained eosinophilic granular casts.

The adrenals, pancreas, and brain appeared normal.

In this instance bilateral papillary necrosis was associated with thrombosis of both renal veins in a child suffering from a respiratory infection.

Case 4.—This boy aged 10 months was well until 12 days before admission when he developed a cough, sneezing, and a running nose. He vomited occasionally at this time, but a week later he began to vomit repeatedly and also had two or three loose motions each day. Micturition was normal. He had convulsions and four distinct fits before admission. On examination on December 7, 1955, he was an ill, grossly dehydrated infant with occasional twitching of the limbs. There was systolic murmur over the heart and the liver was slightly enlarged and smooth. He was transfused with glucose saline and plasma, and penicillin and sulphamezathine were given. Twelve hours after admission he had a further fit and the following day he was limp and dehydrated. The blood pressure fluctuated from 190/120 to 240/150 mm. Hg, and a rogitine test was performed. An unsuccessful attempt was made to control the blood pressure with "ansolysen." The spasticity and convulsions seemed to be controlled by repeated doses of calcium. The initial improvement in his condition was not maintained; he became increasingly oedematous, the liver descended to the umbilicus, free fluid appeared in the abdomen, and he eventually died six days after admission.

Laboratory Findings.—Analyses of urine (per c.mm.) gave the results below:

Date	Red Blood Cells	White Blood Cells	Casts
9/12/55	None	4	0
9/12/55	,,	53	2
10/12/55	,,	3	0

Albumin trace in all specimens, specific gravity varied from 1,008 to 1010. Catheter specimen, no growth on culture.

Blood chemistry gave the following results:

Date	Urea (mg.%)	Sodium (mg.%)	Chlorides (mg.%)	Potassium (mg.%)	Ca (mg.%)
8/12/55	—	270	240	22	—
	Alkali reserve 61 ml. CO_2 per 100 ml. serum				
9/12/55	216	250	—	21	—
10/12/55	188	240	219	19	7·2
	Alkali reserve 57 vol. CO_2 per 100 ml. serum				
12/12/55	135	230	210	15·8	7·8
	Alkali reserve 51 vol. CO_2 per 100 ml. serum				

Necropsy Findings.—Both kidneys were slightly swollen but normal in weight. The cortices were pale and showed a number of small arterial infarcts on the right side and a single small infarct on the left side. The bladder and ureters were normal. The renal veins were patent.

Of the remaining organs, the heart showed considerable left ventricular hypertrophy and a few emboli were found in the arterioles of the lungs.

The right kidney showed coagulation necrosis of a medullary pyramid. This was not sharply demarcated from the cortex but shaded off into areas of necrosis involving the convoluted tubules. In the necrotic pyramid only ghost forms of widely dilated collecting tubules filled with pale-staining amorphous material could be distinguished. The glomeruli, on the other hand, except for areas of recent infarction, were intact. Venous radicles near the boundary zone contained old hyaline thrombi and the interlobular and arcuate arteries showed muscular hypertrophy and intimal thickening. Changes in the left kidney were less severe than those in the right, but a recent arterial infarct was seen in the cortex and some proximal tubules were atrophic or showed degenerative changes.

FIG. 7.—Case 4: necrosis extends to the cortico-medullary junction. Haematoxylin and eosin, × 3.

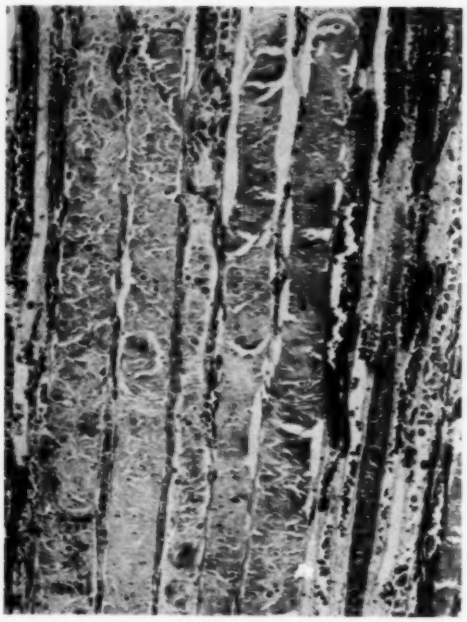

FIG. 8.—Case 4: ghost forms of necrotic tubules separated by vasa recta. Haematoxylin and eosin, × 180.

The adrenals and pancreas appeared normal.

This was a case of unilateral papillary necrosis associated with a varied renal pathology, viz., arterial infarcts, mild hypertensive nephrosclerosis, thrombosis of venous radicles, and a degeneration of the lower nephron.

Discussion

A number of explanations of the pathogenesis of renal papillary necrosis have been suggested.

Friedreich (1877), reporting the condition in a man with prostatic disease and hydronephrosis, thought it was due to urinary stasis giving rise to pressure atrophy, whilst Foulon and Busser (1934) attributed the necrosis in their case to compression of the papillary vessels by a hypernephroma and material used to obtain retrograde pyelograms. Compression of vessels by a "para-amyloid substance" was blamed by Mellgren and Redell (1941). Davson and Langley (1944), however, questioned the role of pressure since the lesion was rarely seen in hydronephrosis or nephrolithiasis.

Some cases of renal necrosis were thought by Günther (1937) to be due to the staphylococcus and *Bact. coli*. Edmondson *et al.* (1947) noted the occasional association of the lesion with tuberculosis and actinomycosis, but they found that *Staph. aureus* was the chief offender, and they emphasized the importance of the necrosin and the coagulase enzyme of that organism. Davson and Langley (1944) suggested that the products of *Bact. coli* might conceivably have a direct toxic action on the renal papillae, but Robbins and Angrist (1949) felt that bacterial toxins alone could not account for the lesion since a similar necrosis was not found in the cortex.

Most authors from Günther (1937) onwards stress the relatively poor blood supply of the pyramids as compared with the rest of the kidney, pointing out that the bulk of the blood reaches the peritubular capillaries after it has passed through the glomeruli and that the pyramids may be particularly vulnerable to ischaemia. Robbins and Angrist (1949) suggested that a reversed Trueta shunt might produce papillary necrosis. Although Trueta, Barclay, Daniel, Franklin, and Prichard (1947) succeeded in diverting blood from the medulla to the cortex, yet papillary necrosis was not produced, suggesting that if a shunt were concerned it could not be the only

factor, and that the papillary tissue was not particularly vulnerable to ischaemia. Nevertheless the anatomical limitation of the lesion to the pyramids and the appearances of the necrotic papillae suggest that ischaemia is concerned in some instances.

The condition has been produced in animals by methods that suggest that toxins, metabolic upset, pressure, or infection may be concerned in its pathogenesis. In 1901 Levaditi produced the lesion by poisoning animals with vinylamine and Rehns by poisoning them with tetrahydroquinoline. Borland and Jackson (1931) reported papillary necrosis in animals kept on a fat-free diet, whilst Muirhead, Vanatta, and Grollman (1950) produced the lesion by ureteral ligation and Robbins, Mallory, and Kinney (1946) observed it in experimental pyelonephritis.

The present series of cases suggests that no single factor is concerned in the pathogenesis of the lesion. Two cases (1 and 2) resembled the picture seen in some adults in that they were associated with pyelonephritis, the appearances suggesting little more than a sloughing off of infected tissue. Abscesses were present in the remaining renal parenchyma, but the papillae had suffered most and this perhaps was not unexpected since the vasa recta loop back to the cortex at this point and infected emboli and bacteria may tend to lodge there.

The remaining two cases were unusual in that papillary necrosis was associated in one instance (Case 3) with renal vein thrombosis, and in the other (Case 4) with a variety of vascular lesions, including thrombosis of the venous radicles and small arterial infarcts. In the absence of any other obvious factor it seemed likely that in both of these cases ischaemia was concerned. In Case 3 the occlusion of the main renal, pelvic, and arcuate veins would presumably affect all the renal parenchyma and therefore the peculiar limitation of the necrosis to the pyramids must be explained. Dilated venous channels in the subcapsular and perinephric tissue suggested that the cortical circulation had been maintained by a collateral circulation similar to that described by Keen (1940) in a case of obstruction of the inferior vena cava by veins piercing the capsule of the kidney. The vasa recta of the pyramidal tissue draining into the occluded arcuate veins cannot have access to this collateral circulation, and the pyramidal tissue must therefore become ischaemic. The part played by renal vein thrombosis in Case 4 can only be a matter of conjecture since the thrombi were small and numerous arterial infarcts were present.

The diagnosis of the condition is extremely difficult and the prognosis bad, but cases have been reported in adults by Alken (1938) and other authors which were diagnosed by retrograde pyelography and, when the lesion was unilateral, treated successfully by nephrectomy. The radiological changes detailed by Günther (1937) and others include dilatation of the tips of the calices, ring shadows in the necrotic papillae, and sloughed off papillae lying free in the renal pelvis. However, the pyelographic changes may not always be helpful and may be indistinguishable from those of pyelonephritis. Few other diagnostic criteria are available and a comparison of the clinical histories and laboratory data of the cases in the present series with one another reveals no one typical pattern, since the primary condition tends to mask the papillary necrosis. Nevertheless in cases of pyelonephritis or renal failure with albumin, pus cells, or red blood cells in the urine the diagnosis of renal papillary necrosis must be considered.

Summary

Four cases of renal papillary necrosis in children are reported.

In two instances the necrosis was associated with pyelonephritis, in a third with renal vein thrombosis, and in a fourth with varied vascular lesions.

The pathogenesis and diagnosis of the condition are discussed, and it is suggested that when the lesion is associated with renal vein thrombosis the survival of the cortical as opposed to the medullary tissue is due to the development of a collateral venous circulation.

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THE PATHOGENESIS OF BRONCHIAL CATARRH AND OF ACUTE AND CHRONIC BRONCHITIS

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"Chronic bronchitis" has recently been discussed from many points of view, all of them important enough, but very little has been said about the aetiology and pathogenesis of the condition. Investigations into the late stages of the disease, clinically, radiologically, or anatomically, do not show the course of the affliction, and, important as it may be to investigate the bacteriology, the effect of antibiotics, or the social conditions, nothing of this kind will elucidate the pathogenetic progress. On the other hand, especially in view of the growing incidence and danger of chronic bronchitis and bronchial carcinoma, it is necessary to investigate in a more detailed way the much neglected field of the normal and morbid anatomy of the bronchi. An attempt is therefore made in this paper to explain the basic pathogenetic factors and not to deal with acute exacerbations and their treatment.

The Normal Bronchus

The general structure of the bronchial tree has recently attracted much attention, particularly the "segmental" structure of the lung. This aspect of pulmonary anatomy will not, however, be dealt with in this paper, which is mainly concerned with the structure of the bronchial wall.

Fig. 1 shows the elements of which the bronchial wall is composed. (The numbering 1–6 is from within outwards.)

(1) The ciliated epithelium is, for the sake of simplicity, represented as a row of oblong, rectangular spaces. The columnar cells are rather spindle-shaped and arranged in two or three layers.

(2) The basal cells form a continuous row of small round cells. They rest on the basement membrane; they are germinal cells and serve as replacements for columnar cells.

(3) The basement membrane is thick or thin according to the bronchial calibre; it is a condensation of the tunica propria tissue (4) and serves as a foothold for the epithelium. The basal cells (3) stick tightly to the membrane.

(4) The tunica propria is a thin layer of smooth connective tissue, rich in vessels; it contains also the elastic fibres. The elements numbered 1, 2, 3, and 4 together form the mucous membrane.

(5) The muscle is arranged in spirals rather than in circles; it acts as the motor of the mucous membrane.

(6) The tunica fibro-cartilaginea consists of strong fibrous and elastic strands and is reinforced by cartilage, decreasing in strength towards the periphery. This layer gives the bronchus its stability and elasticity (Engel, 1947).

The Mucous Membrane

The membrane is normally arranged in a continuous row of folds (Fig. 2). Fig. 2 represents part of a 2.5 mm. bronchus (internal diameter,

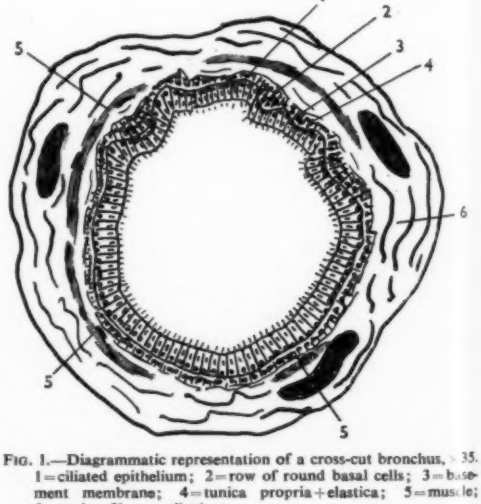

FIG. 1.—Diagrammatic representation of a cross-cut bronchus, × 35. 1=ciliated epithelium; 2=row of round basal cells; 3=basement membrane; 4=tunica propria+elastica; 5=muscle; 6=tunica fibro-cartilaginea.

measured from base to base of epithelium, × 60). The folds are lined by ciliated columnar epithelium and their core consists of tunica propria with elastica. This arrangement used to and may still cause the illusion that the elastica of the bronchi runs in longitudinal strands. This is not so. Both the tunica propria and the elastica surround the bronchus evenly, provided that there are no folds, as can be seen in Fig. 1. In this case the mucosa has only a few shallow folds, and it is quite clear that they contain an excess of tunica propria tissue as compared with the smooth parts of the wall. The folds play an important part in the function and pathology of the bronchus.

Bronchial Catarrh

Bronchial catarrh follows a mild irritation and is not true inflammation. As the name indicates, catarrh is a condition where excess of fluid is a prominent feature. Catarrh is an uncomplicated superficial condition which abates within a few days and is therefore rarely accessible to anatomical investigation. The present author has had the opportunity of studying catarrhal bronchi in the post-mortem room and of carrying out histological examinations. It was evident that, in accordance with clinical observations, the catarrh usually affects only the trachea and main bronchi and stops abruptly at the hilum. Cases of this kind should be diagnosed as "tracheo-bronchial catarrh" and not as bronchitis. There is an abundance of mucus, which is sometimes yellowish, in the lower parts of the trachea and in the main bronchi, and this secretion may derive from the glands, which are particularly numerous in the trachea and main bronchi, attached to the mucous membrane. The epithelium itself may also produce mucus by a dramatic change whereby nearly every columnar cell is turned into a goblet cell. No other changes are seen in uncomplicated cases; there is no infiltration or destruction of mural elements and restitution to normality is the rule.

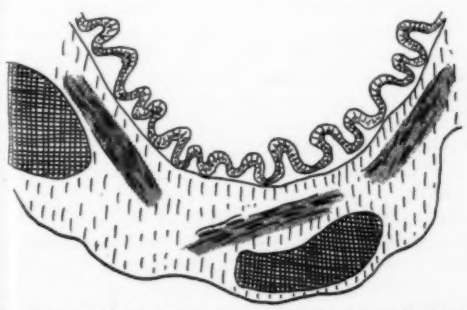

FIG. 2.—Part of a 2.5 mm. bronchus to show the row of folds in which the mucous membrane is arranged, × 60.

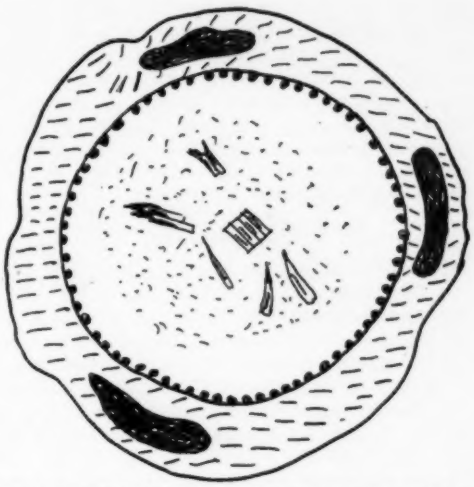

FIG. 3.—Slightly dilated bronchus, the columnar cells of which are cast off whereas the row of basal cells is intact. Mild acute bronchitis.

Bronchial catarrh occasionally occurs in medium and small bronchi. These tubes are less well provided with glands attached to the mucous membrane, and conversion of columnar cells into goblet cells is therefore the main source of mucus. This process can best be observed in cases of recent asthma where catarrh spreads to the small bronchi and bronchioli.

Acute Bronchitis

Acute bronchitis is chiefly localized in medium and small bronchi. The glands are hypertrophic and distended, columnar epithelium is often shed, and some of the dequamated cells may remain in the bronchial lumen mixed up with the secretion and may appear in the sputum (Fig. 3). A profuse quantity of mucus is produced and it may become purulent. The cells either keep their original shape or undergo degeneration and deformation. The basal cells, on the other hand, usually remain tightly adherent to the basement membrane ready for the renewal of lost columnar cells.

Potent infection is the usual cause of acute bronchitis, which may be a serious disease. The wall is deeply affected, generally infiltrated by lymphocytes, plasma cells, or even polymorphonuclears. Deeper layers are sometimes eroded, thus producing the picture of ulcerative bronchitis,

but such destructive conditions are rare and are only mentioned to illustrate the many grades of bronchial inflammation. Severe or repeated attacks of bronchitis soften the bronchial wall with consequent, mostly cylindrical, dilatation. Bronchiectasis may be local or general, temporary or permanent, depending on many factors, such as the intensity of the inflammation or congenital weakness of the bronchi.

Chronic Bronchitis

Only one recent paper describes the morbid anatomical features of chronic bronchitis (Reid, 1954). Ogilvie and Newell (1957) refer only to Reid when discussing the pathology of chronic bronchitis. Reid is mainly concerned with late changes of the bronchioli rather than of the bronchi, arguing that obliterative bronchiolitis is the main cause of chronic bronchitis. She speaks of mild cases without describing the criteria of "mild" and her "advanced" cases obviously represent final stages with variegated secondary changes in the bronchioli and lung tissue. In mild cases hypertrophy of the mucus-secreting elements, glands and goblet cells, is noted. As conversion of columnar cells into goblet cells has been observed by the present author in catarrh, Reid's mild cases seem to be of catarrh rather than of bronchitis. There are of course no definite boundaries between catarrh and mild bronchitis, so that hypertrophy of goblet cells may also be a sign of mild bronchitis.

Pathogenetic Factors.—The condition affects mainly people of middle or old age. It is rampant in industrial areas, where the incidence and death rate are terrifying. Figures published from Leeds (Edwards, Buckley, Fear, Williamson, and Zinnemann, 1957) illustrate the severity of the problem. According to these authors the mortality in one year compared with that of pulmonary tuberculosis and bronchial carcinoma is as follows:

Pulmonary tuberculosis	67 cases
Bronchial carcinoma	270 cases
Chronic bronchitis	463 cases

Common experience shows that chronic bronchitis usually develops after repeated or protracted attacks of acute bronchitis; emphysema with rigidity of the dilated thorax (barrel chest) is often an additional if not the primary pathogenetic factor. How far atmospheric conditions or congenital susceptibility should be accused will not be discussed.

Repeated infections of the bronchial tree, which are so common a feature in chronic bronchitis, are not likely to be the cause of the disease, but rather they are conditioned by some change or susceptibility of the bronchi. Observations in early cases of repeated acute bronchitis suggest the following course. Repeated or protracted attacks of acute bronchitis, local or general, lead to slight cylindrical bronchiectasis, and this change is the forerunner of further attacks of bronchitis which will maintain or even increase the bronchial dilatation.

Slight cylindrical bronchiectasis produces important changes in the arrangement of the bronchial mucosa. As has been shown in Fig. 2, the mucous membrane is normally arranged in folds which serve a double purpose. It is evident that the membrane is larger than the internal bronchial circumference. Therefore the folds permit the respiratory movements of the bronchi without overstressing the membrane in the inspiratory widening of the bronchial lumen, and, secondly, they increase the surface of the membrane. The folded membrane is, as a simple matter of common sense, more efficient than a smooth lining for arresting and dealing with dust and bacteria suspended in the air. Bacilli caught in the folds are not only stopped there and expectorated, but in addition deprived of their virulence and/or destroyed. That this is so can be inferred from the fact that normal pulmonary tissue is practically aseptic. This fact is the more startling as not all bacteria are arrested and expectorated, as can be concluded from the pathogenesis of primary tuberculosis. Therefore reduction of virulence is obviously the explanation of the fact that pulmonary tissue is aseptic in normal cases.

The folds are necessarily flattened in dilated bronchi, and the mucous membrane now adheres tightly to the bronchial wall. It might even be worse because the membrane will to some extent degenerate owing to the pressure in inspiration, which normally is rendered ineffective because the folds give way by flattening. The antibacterial function of the mucosa is likely to be handicapped in dilated bronchi and acute exacerbations will easily occur. The many attacks of acute bronchitis in chronic cases are explicable in this way. A vicious circle will gradually develop, because each attack will contribute to the damage done to the mucosa and will maintain or increase the bronchial dilatation. Finally the effect of chronic bronchitis affects the general condition of the subject, and especially the heart, leading to the dangerous condition of cor pulmonale. The secondary changes of chronic bronchitis which gradually lead to the fatal outcome are not the subject of the present paper.

Conclusions and Summary

Chronic bronchitis is not the result of repeated attacks of acute bronchitis ; they are not the cause of the disease, but are consequent on primary changes in the bronchial wall, especially in the mucous membrane. The condition begins with slight cylindrical bronchiectasis due to attacks of acute bronchitis or is a concomitant of emphysema. Dilatation of the bronchi leads to disarrangement and functional disorder of the mucous membrane. These changes pave the way for further attacks of acute bronchitis, which damage the mucosa, and bronchial dilatation. Eventually a vicious circle develops and the condition grows worse until the bronchial disintegration is irreparable and secondary changes in the bronchial tree and in the lung tissue complicate the situation, affect the general condition, and lead to cor pulmonale.

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FIBRINOLYSIS IN NORMAL PREGNANCY

BY

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It is well known that a low level of circulating fibrinogen may be associated with bleeding in a variety of obstetric complications, as, for example, accidental haemorrhage. The actual cause, however, of the depletion of fibrinogen in these conditions is debatable (Moore, 1955), but one feature of such states is the frequent presence of a circulating proteolytic agent which is capable of destroying fibrin. It is possible that this agent is responsible for the bleeding, though attempts to demonstrate it have not always been successful.

One explanation of the failure to detect lysis is suggested by the work of Fearnley, Revill, and Tweed (1952), who showed that plasma fibrinolytic activity is thermolabile. This activity is quickly destroyed at room temperature, but can be preserved by keeping the plasma at ice temperature.

Such a precaution has been adopted in the present work, which is concerned with the behaviour of plasma fibrinolytic activity in normal pregnancy, labour, and the puerperium.

Methods and Material

Qualitative Test (Modified from Fearnley and Tweed, 1953).—Bottles and syringes are carried to and from the wards in a container filled with ice. Further manipulations in the laboratory are carried out using ice-cold glassware and ice-cold reagents.

Venous blood is taken into a bottle containing sequestric acid as an anticoagulant and carried immediately to the laboratory. The blood is centrifuged at 3,000 r.p.m. for two minutes. Doubling dilutions of the plasma up to 1/16 are made with a veronal buffer solution (*p*H 7.35) containing merthiolate in a final concentration of 1/100,000. The neat plasma and dilutions are clotted immediately with sufficient thrombin to produce a clot in 30 seconds and placed in a water-bath at 37° C.

The test is read after 24 hours' incubation. If a clot is absent in any one tube the test is regarded as positive.

Semi-quantitative Test (Modified from MacFarlane and Pilling, 1946).—Doubling dilutions of the test plasma are made from 1/8 to 1/2,048. The diluent consists of veronal merthiolate buffer containing fibrinogen in an approximate concentration of 50 mg.%. The first tube (1/8) is a dilution of plasma with veronal merthiolate buffer only; the concentration of the fibrinogen in this tube would lie between 40 mg. and 60 mg.%. In this way the concentration of fibrinogen is the same in all tubes, i.e., approximately 50 mg.%.

The tubes are clotted with thrombin as before and placed in a water-bath at 37° C. The whole procedure of the qualitative and semi-quantitative tests up to incubation is carried out within 15 minutes from the obtaining of the blood.

The tubes are examined after 24 hours' incubation and the titre expressed is the dilution of the last tube in which a clot is absent. Some tubes at a higher dilution show partial dissolution of the clot, but, since it is difficult to assess the degree of lysis, these tubes are not included in the titre expressed.

Repeated bacteriological tests demonstrated that the tubes of both tests remained sterile.

Reagents.—The following are required:

Veronal Buffer Solution (*p*H 7.35).—Sodium diethyl barbiturate 12.75 g., 5.67 g. sodium chloride, 430 ml. 0.1 N HCl, and 570 ml. distilled water.

Thrombin.—Thrombin (Roche) is dissolved in distilled water at a concentration of approximately 10 units/ml. and stored frozen. It is thawed immediately before use.

Fibrinogen.—The method of preparation is modified from that of Ware, Guest, and Seegers (1947). Fresh human plasma (sequestrene anticoagulant) is left standing at room temperature for several hours. It is then frozen overnight at −10° C. The following morning it is allowed to thaw slowly until a piece of ice the size of a grape remains. The plasma is spun quickly at 3,000 r.p.m. At the end of spinning a small piece of ice should still be present. The supernatant is discarded. The sediment includes fibrinogen and is washed once with ice-cold saline by centrifuging for one minute at 2,000 r.p.m. at room temperature. The supernatant is discarded and the precipitate dissolved in normal saline at 37° C. The solution is centrifuged for two minutes at 2,000 r.p.m., decanted, and freeze dried. Simple experiments demonstrated that this preparation of fibrinogen possesses neither fibrinolytic nor anti fibrinolytic activity in the concentration used.

The patients from whom test samples were obtained were the ordinary out-patients and in-patients of the hospital. About 500 patients were examined by the qualitative technique and 400 of these were also tested by the semi-quantitative method. At the time of veni

puncture all patients were normal. Of those examined during the antenatal period, over 90% remained normal and those examined during labour and the puerperium had had a normal pregnancy. Sequential tests on individual patients were not carried out.

Fifty-four normal non-pregnant women were tested as a control series.

Results

Qualitative Test.—The percentage incidence of lytic activity in non-pregnant females, at all stages of pregnancy, during labour, and the puerperium is given in Table I and Fig. 1.

The figure for non-pregnant females, i.e., 92%, corresponds closely to that of Fearnley and Tweed (1953), who, using a similar technique, found lysis in 50 out of 60 patients.

TABLE I
INCIDENCE OF FIBRINOLYSIS IN NORMAL PREGNANCY

Group		Total No. of Patients	No. of Patients Showing Lysis
Non-pregnant		54	50 (92·6%)
Gestation Period in Weeks	0 to 10	23	23 (100%)
	11 to 15	43	40 (93%)
	16 to 20	52	38 (73%)
	21 to 25	23	11 (47·8%)
	26 to 30	26	5 (19·2%)
	31 to 35	40	3 (7·5%)
	36 to labour	53	6 (11·3%)
Labour	First and second stages	54	4 (7·4%)
	Third stage	7	2 (29%)
Puerperium	0 to 3 hours	31	24 (77·4%)
	3 hours to 8 days	93	83 (89%)
	6 weeks	13	11 (85%)

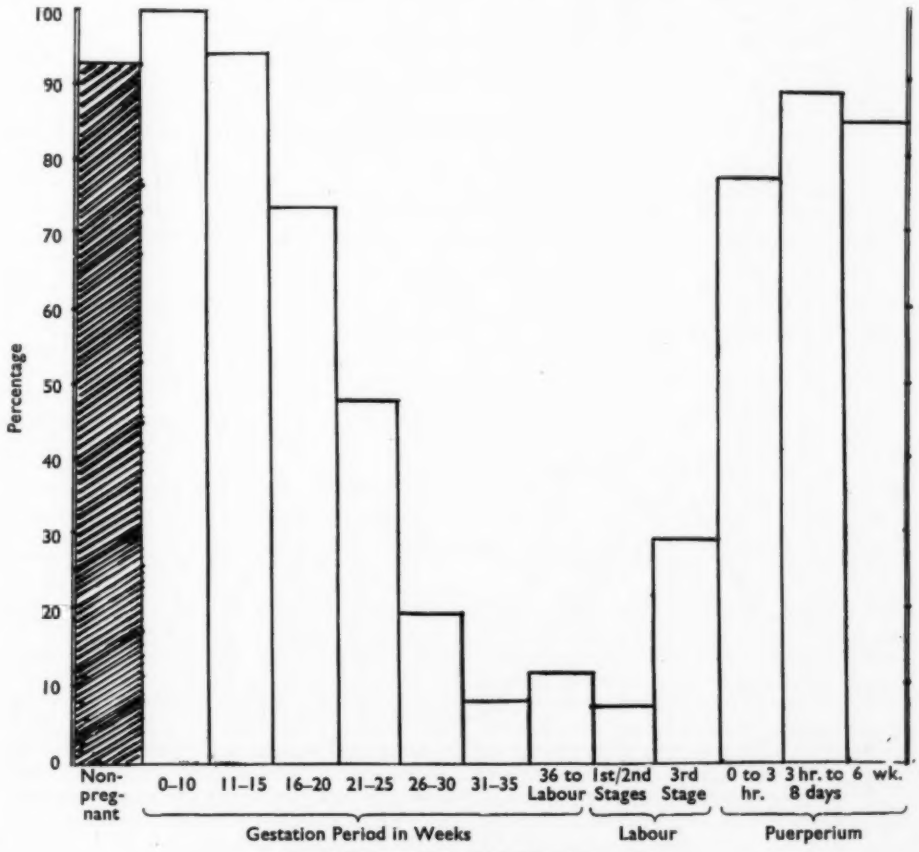

FIG. 1.—Incidence of fibrinolysis in normal pregnancy.

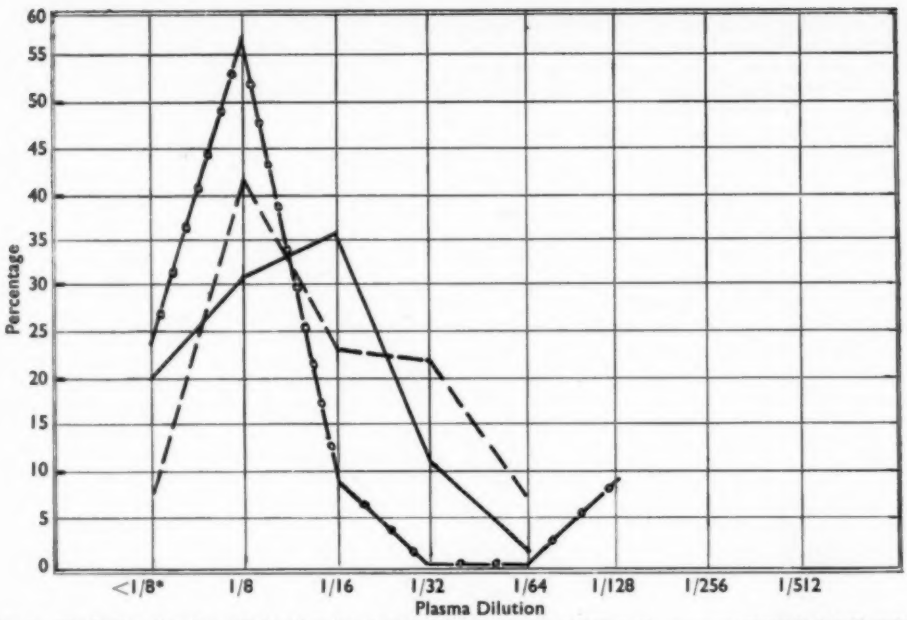

FIG. 2.—Distribution of semi-quantitative titres in antenatal period. ——— non-pregnant. – – – – – antenatal : 0 to 20 weeks. —o—o—o antenatal : 20 weeks to labour. *≡ < 1/8 ≡ no dissolution of clot in the lowest dilution, but qualitative test is positive.

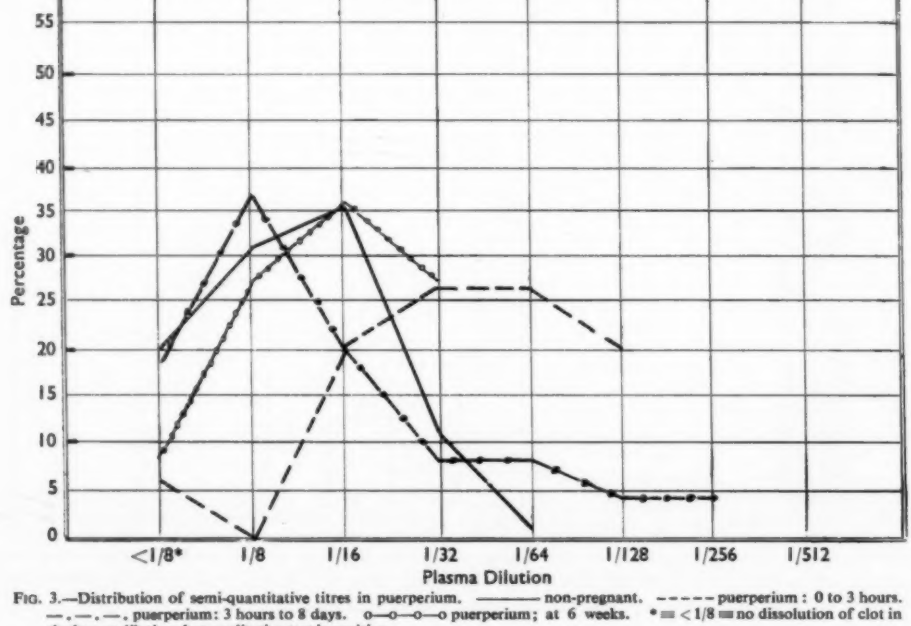

FIG. 3.—Distribution of semi-quantitative titres in puerperium. ——— non-pregnant. – – – – – puerperium : 0 to 3 hours. —.—.—. puerperium : 3 hours to 8 days. o—o—o—o puerperium; at 6 weeks. *≡ < 1/8 ≡ no dissolution of clot in the lowest dilution, but qualitative test is positive.

TABLE II
DISTRIBUTION OF SEMI-QUANTITATIVE TITRES IN ANTENATAL PERIOD AND PUERPERIUM

Group	Total No. of Patients	No. of Patients Showing Lysis	Distribution of Titre							
			< 1/8*	1/8	1/16	1/32	1/64	1/128	1/256	1/512
Non-pregnant ..	48	45	9 (20%)	14 (31·1%)	16 (35·6%)	5 (11·1%)	1 (2·2%)	—	—	—
Gestation period in weeks:										
0 to 20 ..	118	101	7 (6·9%)	42 (41·6%)	23 (22·8%)	22 (21·8%)	7 (6·9%)	—	—	—
20 to labour ..	117	21	5 (23·8%)	12 (57·2%)	2 (9·5%)	—	—	2 (9·5%)	—	—
Puerperium:										
0 to 3 hours ..	20	15	1 (6·6%)	—	3 (20%)	4 (26·7%)	4 (26·7%)	3 (20%)	—	—
3 hours to 8 days	55	49	9 (18·4%)	18 (37%)	10 (20%)	4 (8·2%)	4 (8·2%)	2 (4·1%)	2 (4·1%)	—
6 weeks ..	13	11	1 (9·0%)	3 (27·3%)	4 (36·4%)	3 (27·3%)	—	—	—	—

* ≡ < 1/8 ≡ no dissolution of clot in the lowest dilution, but qualitative test is positive.

The most interesting finding is the progressively falling incidence of lysis found after the 15th week of pregnancy, reaching the lowest value of about 10% during the last 10 weeks and the first two stages of labour. Equally startling is the rapid increase of incidence after delivery so that the percentage value three hours postpartum is about 75% and at 24 hours reaches a normal non-pregnant value of 90%. This value remains unchanged up to seven days and at six weeks postpartum.

Semi-quantitative Test.—It is realized that this test cannot be anything more than semi-quantitative, for although fibrinogen and thrombin remain relatively constant, plasma factors other than the fibrinolysin are also diluted. Nevertheless the results within the individual groups are so consistent as to suggest that the different titres do, in fact, represent different levels of lytic activity.

The actual test also behaved consistently. For example, in only six instances (out of about 400 estimations) did it occur that, when lysis was present in a high dilution, lytic activity was absent in tubes of a lower dilution.

The range of titre of lytic activity for non-pregnant women during pregnancy, labour, and the puerperium is given in Table II and Figs. 2 and 3. The important conclusions to be drawn are:

(*a*) The highest titre in the non-pregnant group is 1/64 and 98% of cases do not exceed 1/32. The values for the antenatal group of up to 20 weeks' gestation are about the same, i.e., a highest titre of 1/64, with 93% of cases not exceeding 1/32. In the group 20 weeks to labour, although the highest titre is 1/128, about 92% of cases have a value not exceeding 1/16. This would suggest that not only is the percentage incidence reduced during the last 20 weeks of pregnancy (Fig. 1) but that, when lysis is demonstrable, it tends to have a lower titre. Such a conclusion can only be tentative because of the fewer positive cases in the later weeks of pregnancy.

(*b*) It is apparent from Fig. 3 that the distribution of the titres at six weeks postpartum is the same as in the normal non-pregnant group. However, during the period three hours postpartum to eight days postpartum the highest titre reaches 1/256 with 84% of values less than 1/32. There is a suggestion, therefore, of a slight increase in activity although most cases fall within the normal range.

Nevertheless it is the distribution curve for the period 0 to three hours postpartum which shows the most obvious increase of titre. In this group, composed of only 15 cases, the range reaches 1/128, but seven cases, i.e., 47%, have a titre of 1/64 or more.

Of the 40 patients who were tested during labour, only two showed lysis and these gave titres of less than 1/8 and 1/16.

Discussion

Recent reports concerned with fibrinolysis in pregnancy give widely divergent results. Cervini and Ficola (1951), Margulis, Luzadre, and Hodgkinson (1954), and Niesert (1955) agree that there is a greater incidence of fibrinolysis in the puerperium than in the antenatal period. Ciculla and Luraschi (1953a and b) came to the opposite conclusion. These latter authors also investigated patients at different periods of gestation but unfortunately did not give details. Shea (1955) investigated fibrinolytic activity in 34 puerperal women and found activity in only two, and somewhat earlier MacFarlane and Biggs (1946) mention that they found no evidence of proteolytic activity in 137 normal women during pregnancy. None of these reports can be said to agree wholly with the present findings. The reason for the discrepancy is probably technical, for none of the above authors preserved lytic activity by keeping the plasma and reagents at ice temperature.

The results reported here are surprising, particularly those obtained during labour. It has been demonstrated that both exercise and stress increase lysis in normal men and women (Biggs, MacFarlane, and Pilling, 1947; Truelove, 1953). Labour may be assumed to involve both exercise and stress and yet lytic activity at the time is exceptional. Whether this inhibition, in the antenatal period as well as during labour, is due to a low concentration of fibrinolysin or to an increased antifibrinolysin is unknown and is being investigated at the present time.

The effect of fibrinogen concentration on lytic activity has been commented on by various workers. Apparently fibrinolysis *in vitro* decreases with an increase in the amount of fibrinogen (Shulman, 1952a; Bozzo, Piomelli, and Schettini, 1956). It is possible, therefore, that the high levels of plasma fibrinogen which may be found in pregnancy could explain the inhibition of lysis. However, in the puerperium, normal or high fibrinogen levels are associated with normal or enhanced lytic activity, and Shulman, too (1952b), found that fibrinogen levels *in vivo* had no influence on fibrinolytic activity.

Clearly many factors could have an influence, but two may be mentioned. Lipaemia, which is known to be increased in pregnancy, has been shown to exert an anti-fibrinolytic effect in men and non-pregnant women (Greig, 1956); and hormones, too, exert an influence on lysis in the experimental animal (Ungar, Damgaard, and Hummel, 1951). There is no definite evidence to link either of these two factors with fibrinolytic activity in pregnancy and their role can be no more than speculative.

Finally there is one practical point of importance. Whenever lytic activity is discovered in the puerperium it is often assumed that such activity is abnormal. It is clear from the present work that lytic activity in the puerperium is a return to the normal physiological state of the non-pregnant woman and that therefore some kind of quantitative assessment is necessary in order to determine abnormal degrees of lysis.

Summary

Qualitative and semi-quantitative assessments of fibrinolytic activity in normal pregnancy, labour, and puerperium and in a number of non-pregnant women have been carried out.

The results show a marked decrease in the activity in the later months of pregnancy and labour and a quick return to about the non-pregnant level immediately after delivery.

One of us (J. J. Biezenski) receives a wholetime grant from the Medical Research Council of Ireland and we are much indebted to them. We wish to thank the Master, medical and nursing staff of the Rotunda Hospital for their willing co-operation, and E.H.Thornton, of Trinity College, Dublin, for statistical advice.

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DIRECT COMPATIBILITY TESTING

BY

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Despite the rapid development of knowledge it still remains fundamental that mistakes within the ABO and Rhesus systems are responsible for virtually all fatal mismatched transfusions. It therefore cannot be too highly stressed that the careful preliminary grouping of the bloods of all patients for transfusion is of prime importance, irrespective of whether there is a previous record of their blood group or not. This should always be regarded as an essential part of any cross-matching routine, and in urgent cases Stratton's (1955) " sandwich technique " is highly recommended for rapid Rh typing.

The " emergency crossmatch " presents a problem, and the vital question which needs answering is: What can safely be accepted as the minimum incubation period which will not impair the efficiency of the particular compatibility tests being used ?

The concept of gradual red cell sensitization progressively increasing with time is fallacious. Antigen-antibody union takes place quite rapidly, and, after a relatively short time, which varies slightly with different methods, further prolonged incubation does not increase the sensitivity or render the detection of very weak antibodies significantly more likely. This is shown by the investigations which are detailed later, and the results obtained by Lewis and Chown (1957) using a 10-minute albumin agglutination technique substantiate this view.

The ideal cross-matching technique should provide a simple, easily performed procedure which is not only highly sensitive but which at the same time will yield reliable results when used routinely.

Certain practical points have been investigated, and on the basis of the findings obtained a simple direct matching procedure, which is not time-consuming but still combines the advantages of both trypsin and Coombs testing, is recommended.

The Indirect Coombs Test (A.H.G.)

Minimum Incubation Period for Reliable Results.—Twenty-five sera were tested in triplicate, all tests being standardized so that 4 vol. of serum was used to sensitize 1 vol. 50% suspension of washed packed Group O R_1r cells. Incubation periods of 10, 30, and 120 minutes were used. The test sera ranged in potency from extremely weak ones, giving only a trace albumin agglutination, to strong sera with albumin titres up to 1/128.

Readings were made by the usual tile method and the reactions timed with a stopwatch.

If no agglutination had occurred at the end of seven minutes a negative result was recorded.

All tests gave positive results although some of the " 10-minute incubation " ones were rather weak. The usual reaction in these cases was a fine granularity first appearing between two and four minutes, gradually becoming clear cut, but still tending to retain a homogeneous pink background at the end of seven minutes.

The reactions of the " 30-minute incubation tests " were all strong and clear cut. They were as good as those obtained by longer incubation and in a surprising number of instances they were somewhat better defined than the corresponding two-hour agglutinations.

There seems little doubt that the optimal incubation time for complete sensitization is much shorter than is generally appreciated. Using the indirect Coombs for direct compatibility testing, it is considered that an incubation time of 30 minutes is adequate. In an emergency, a 10- or 15-minute Coombs test is an extremely valuable procedure which is capable of detecting very weak sensitization.

Effect of Variable Serum-cell Ratios on Sensitivity of Coombs Reaction.—The Coombs reactions obtained after sensitizing red cells for a standard incubation period of 30 minutes using serum : cell

ratios varying from 2 : 1 to 10 : 1 have shown no significant differences. It is therefore apparent that, although theoretically it might be expected that the addition of a greater volume of serum would increase the amount of antibody available for each red cell, in practice the effect is minimal. The probable explanation of this is the fact that, although the total amount of antibody is thereby increased, the actual concentration of antibody molecules per unit volume of serum remains precisely the same.

A Modified Tube Technique.—With the development of a simplified trypsin-saline and trypsin-Coombs compatibility routine in mind, the results of the Coombs test performed in tubes were compared with those obtained by the more generally adopted tile method. For this comparative trial a series of very weak albumin agglutinating anti-Rh sera was used. A parallel series of tests was made by the tile method and by a modified tube technique described here.

Method.—Unit volumes of 10% washed test cell suspensions were sensitized with 4 vol. of serum. Tests were set up in triplicate for incubation periods of 10, 30, and 120 minutes respectively. After incubation they were washed three times in saline, the final supernatant being completely removed so as to leave a dry button of red cells. Three drops of suitably diluted A.H.G. reagent were added to each tube. The tubes were vigorously shaken to mix the cells, allowed to stand at bench temperature for two minutes, and then centrifuged at 1,500 r.p.m. for two minutes. Readings were made by gently resuspending the cells and microscoping if necessary.

Results.—In most instances the results by tile and tube techniques were similar, but in some cases the tube test gave clearer results and was distinctly more sensitive for the detection of minimal sensitization. The results obtained in four antenatal patients whose sera contained very weak antibodies are shown in Table I, and it will be noted that in one case the tile test was negative whereas the tube test was weakly positive. In sera 2 and 3 the tile method was negative at 10 minutes whereas the tube showed positive agglutination after this period of incubation.

TABLE I
RESULTS IN TILE AND TUBE TESTS ON FOUR ANTENATAL SERA

Serum No.	Method	Incubation Time		
		10 Min.	30 Min.	120 Min.
1	Tile	Uniform fine granularity	+++	+++
	Tube	++	++++	+++
2	Tile	Neg.	Neg.	Neg.
	Tube	Weak +	Weak +	Weak +
3	Tile	Neg.	Very weak granularity	Weak +
	Tube	Weak +	+++	++
4	Tile	Neg.	Doubtful +	Weak +
	Tube	+	+++	++

Further experience in a large number of controlled tests has led to the conclusion that for routine usage the tube technique gives more consistent results and is distinctly more sensitive than the tile method. There does not appear to be any tendency to false agglutination and in all cases the negative controls have shown no trace of agglutination.

Stability of Stored Diluted A.H.G. Serum.—The instability of A.H.G. serum and its inability to withstand repeated thawing and refreezing are well-recognized drawbacks which can cause anomalous results. Mollison (1951) states: "Attention must be drawn to the fact that anti-human globulin sera diluted in saline lose their potency very rapidly even when stored at – 20° C. The diluted reagent should be prepared freshly each day. . . ." This has not been the author's experience nor that of his colleague Dr. F. Marsh (1957), to whom he is indebted for the results of a two-year trial using stored diluted antiglobulin sera. Diluted serum stored at – 20° C., placed in a water-bath for a couple of minutes and used for testing, then immediately replaced in the refrigerator, will withstand thawing for at least seven times without significant loss of its ability to agglutinate weakly sensitized red cells. Such diluted reagent has been kept and used regularly once daily for periods up to 10 to 14 days without deterioration. Reagent made up and used at weekly intervals remained stable and still strongly agglutinated sensitized cells when finally tested for the seventh time after being stored for eight weeks.

Initially storage results were not constant or reliable, and it was noted that odd A.H.G. sera would deteriorate much more rapidly and unexpectedly, as Mollison states. Experience showed that these failures were attributable to two causes. In the first place, the extreme importance of avoiding any trace of globulin contamination in the saline used as the diluent had not been sufficiently appreciated. Secondly, it was found that if the A.H.G. serum from which the diluted reagent was made had itself began to deteriorate, then the diluted reagent would be quite unstable. Provided meticulous care is taken in the preparation of the diluted reagent from a fully potent A.H.G. serum which has not started to deteriorate, then this reagent can be stored at – 20° C. for long periods. It will withstand thawing up to at least seven times; nor does there appear to be any loss of specificity and false agglutinations have not been encountered. The great advantage and convenience of having a

reliable reagent immediately available for use, particularly for night emergencies, can be appreciated.

Storage of Sensitized Cells Suitable for Coombs Control.—Washed, packed, sensitized red cells may be stored at 6° C. in twice their volume of 0.5% formol-saline solution (1.25 ml. formaldehyde diluted to 100 ml. in saline). The cells retain their freshness and give clear-cut Coombs reactions even after a week's storage. This simple practical procedure provides a suitable Coombs control available for any emergency.

Albumin Tube Tests

The rapidity with which agglutination takes place in Stratton's (1955) sandwich test clearly indicates that prolonged incubation is not essential. The short albumin method advocated by Lewis and Chown (1957) gives excellent results, but since it seemed that the important factor here might well be the high-speed centrifugation, a parallel series of "layered albumin tests" centrifuged after 10 minutes' and two hours' incubation respectively were compared with Lewis and Chown's method (Table II). The results clearly indicate that the 10-minute tests give equally good results compared with the longer incubation, and in fact in one instance (No. 15) the 10-minute test was definite whereas the corresponding two-hour test was doubtful.

The agglutinations in the "layered albumin tests" were not always quite so strong or clear cut as those obtained by Lewis and Chown's method. It is thought that this may be due to the fact that the final bovine albumin concentration in their method is 30% as compared to 20% in the layered albumin (the diluent effect of serum excluded). It would seem therefore that, if albumin is used for compatibility testing, a 10-minute centrifugation test is to be preferred, since the results of tests on a wide selection of sera have clearly indicated that inhibition of agglutination by "zoning antibodies" is unlikely, and furthermore it is equally if not more sensitive than the usual two-hour incubation test.

TABLE II

COMPARATIVE RESULTS OF LAYERED ALBUMIN AND THE SHORT ALBUMIN METHODS

Serum	Antibody Titre		Layered Albumin		Short Albumin (Lewis and Chown, 1957)
	Saline	Albumin	10 Minutes	2 Hours	
1	64	16	4	3	4
2	16	8	2	2	3
3	64	32	4	3	3
4	256	512	4	4	4
5	16	4	4	3	4
6	Nil	32	4	3	4
7	,,	8	1	2	3
8	,,	64	3	2	3
9	,,	4	4	3	4
10	,,	8	3	2	3
11	,,	4	3	2	4
12	,,	2	4	2	3
13	,,	1	3	2	2
14	,,	< 1	1	M+	3
15	Minimal sensitization		M+	M±	2

Key: 4 = Single clump. 3 = 3–4 large agglutinates. 2 = several agglutinates. 1 = Numerous fine agglutinates. M = Microscopic reading.

The Trypsin-saline Test

For technical details the reader is referred to a previous article in this journal (Young, 1957). Further experience has shown that the 1.2% buffered trypsin reagent is quite stable and will store well at 6° C. for four to five days, which is advantageous since it permits one to keep the reagent ready for use at night.

The results obtained by centrifugation after 20 minutes' incubation have been completely reliable in well over 1,500 cases. However, it is stressed that after 10 minutes this is not so. A subsequently performed trypsin-Coombs reaction is extremely sensitive and may be positive when both trypsin-saline and albumin tests are negative (Table III, serum 18).

The reliability of the albumin, trypsin-saline, trypsin-Coombs, and indirect Coombs compatibility tests after varying incubation periods are well shown in Table III. It should be particularly noted that in the group of cases with minimal sensitization the only completely reliable tests at 10 and 30 minutes are the trypsin-Coombs and indirect Coombs. In all other cases read at 30 minutes (with the exception of No. 16) the trypsin-saline test was positive, and all cases except the minimal sensitization group gave a positive 10-minute albumin centrifugation test.

A Simple Practical Direct Matching Routine with a Suggested Procedure for Emergency Cross-matching

The saline agglutination test at room temperature has been excluded for two reasons. Non-specific and specific cold agglutinins are encountered quite frequently and often cause unnecessary anxiety which may result in the rejection of the donor blood. Although their agglutinins react more strongly at lower temperatures, ABO incompatibility can be detected easily in the 37° C. tubes when these are centrifuged at high speed before the final readings are made. For "good risk recipients," i.e., patients without a previous history of transfusions, pregnancies, or miscarriages, the cross-matching procedure does not need to be so comprehensive as for "bad risk cases" whose sera may contain rare immune antibodies, such as anti-Fy, which is only detectable by the Coombs

TABLE III
COMPARATIVE RESULTS OF COMPATABILITY TESTS AFTER VARYING INCUBATION PERIODS

Sera	10 Min.				30 Min.				120 Min:			
	Albumin	Trypsin-saline	Trypsin-Coombs	Indirect Coombs	Albumin	Trypsin-saline	Trypsin-Coombs	Indirect Coombs	Albumin	Trypsin-saline	Trypsin-Coombs	Indirect Coombs
Group I. Albumin titres over 1:32												
1	++	−	++	++	++	+	+++	+++	++	+	+++	+++
2	+++	++	+++	+++	+++	+++	++++	++++	+++	+++	+++	+++
3	+	+	++	++	+	+++	+++	+++	+	++	+++	+++
4	+++	±	++	++	+++	+	++++	++++	++	+	+++	+++
5	++	+	+++	+++	++	+++	++++	++++	++	++	+++	+++
Group II. Albumin titres 1:4 to 1:16												
6	++	±	++	++	++	++	+++	+++	++	++	+++	+++
7	++	+	++	+	++	++	+++	+	+	++	+++	+
8	++	+	++	+	++	++	+++	++	++	+++	+++	++
9	+	±	++	++	++	++	+++	+++	++	++	+++	+++
10	+	−	+	+	++	+	++	++	++	+	++	++
11	++	±	+	+	++	+	+++	+++	++	+	+++	+++
Group III. Albumin titres less than 1:4												
12	+	−	++	++	+	+	++++	+++	+	++	+++	++
13	+	−	+	+	+	M+	++	++	+	+	++	++
14	++	±	++	++	++	+	+++	++	++	++	+++	++
15	++	+	++	++	++	++	+++	+++	++	++	+++	+++
16	+	−	+	+	+	−	+	++	+	−	+	+
Group IV. Minimal sensitization												
17	±	−	+	+	+	±	++	++	+	+	++	++
18	−	−	+	+	−	−	+	+	−	−	+	+
19	+	−	++	++	+	±	++	++	+	±	++	++
20	±	±	+	+	±	+	++	++	±	+	++	++

test. A direct matching routine is presented which is suitable for both classes of recipient. Emergency cross-matching is given special consideration.

Reagents for Trypsin-saline Test.—For further details the reader is referred to a previous publication (Young, 1957).

Stock Trypsin Solution.—A solution is made up of 3% crystallized trypsin (Armour Laboratories) in N/20 HCl. This remains stable for four to six weeks stored in the refrigerator.

Buffered Trypsin for Tests.—A 1.2% solution is made from the stock trypsin using *p*H 7.7 phosphate buffer solution as diluent. This dilute solution will remain stable for a few days if stored in the refrigerator.

Stored Sensitized Coombs Control Cells.—This control can be stored up to a week in 0.5% formol-saline.

Stored Diluted A.H.G. Serum.—As discussed previously.

p*H* 7.7 *Phosphate Buffer :*

A. $Na_2HPO_4.12H_2O$ .. .. 4.11 g.%.
B. $NaH_2PO_4.2H_2O$ .. .. 2.34 g.%.
Mix 90.5 parts of A with 9.5 parts of B for use.

Direct Matching Routine.—Separate patient's serum and cells. Wash the cells twice and determine ABO and Rh groups. For ABO grouping a tube technique is preferred. The Rh group can be determined by the trypsin-saline method.

Wash donor cells twice in saline and make up to a 10% suspension. For each donor blood to be given set up the following two tube-tests and incubate them in a water-bath at 37° C. for one hour.

(1) *Trypsin-saline Test.*—Two volumes patient's serum plus 1 vol. 10% donor cells plus 2 vol. 1.2% trypsin solution.

(2) *Saline Agglutination Test.*—Four volumes patient's serum plus 1 vol. 10% donor cells.

Centrifuge both at 1,500 r.p.m. for one minute. All doubtful or negative readings are checked microscopically.

In all antenatal patients, in women with previous pregnancies or abortions, and in any patient who has had a previous transfusion or is suffering from a haemolytic disorder, the Coombs and trypsin-Coombs tests should now be carried out if the trypsin-saline test is negative.

Wash the cells in tubes (1) and (2) three times in saline, finally pipetting off the supernatant saline completely leaving a dry button of cells. To each tube add 3 drops of a suitably diluted A.H.G. serum which has been checked against the stored sensitized Coombs control cells. Shake vigorously and stand at bench temperature for two minutes. Centrifuge at high speed for two minutes. Check readings microscopically if doubtful.

Emergency Cross-matching.—(1) Check patient's ABO group by the tile technique.

(2) Rapid Rhesus typing by Stratton's (1955) sandwich test is recommended.

(3) Set up tubes (1) and (2) as in the routine method. Incubate for a minimum of 10 minutes, preferably 20 minutes whenever possible. Read microscopically and always perform Coombs tests if negative.

(4) While the above tubes are incubating perform a 10-minute layered albumin test using a 50% saline suspension of donor cells thus: into a 3 × 3/8 in. test-tube place 3–4 vol. patient's serum, 1 vol. 50% donor cells, 2 vol. 30% bovine albumin. Shake vigorously for a minute and then allow to stand at bench temperature for 10 minutes. Centrifuge at 1,500 r.p.m. for two minutes. Read by rapid rotation of the tube when the packed cells will disperse into the supernatant if negative. Most positives give large solid clumps unless the antibody is very weak, in which case the agglutinates are finely dispersed. This is a valuable procedure as is clearly shown by the results given in Table II.

Summary

Following a discussion of certain practical points regarding the storage of diluted A.H.G. serum and Coombs positive control cells, which enables these to be kept immediately available for use in an emergency, a modified tube technique for the routine performance of Coombs tests is recommended as giving more reliable and sensitive results than the usual tile method.

The reliability of different compatibility tests after short incubation periods has been investigated and comparative results are shown in Table III.

Results are given in Table II which indicate clearly that a " 10-minute centrifuged albumin test " is a valuable emergency procedure.

The introduction of the highly sensitive trypsin-saline test for direct matching is advocated and procedures for both routine and emergency compatibility testing are given.

I wish to acknowledge my gratitude to my colleagues, Dr. F. Marsh and Dr. I. Tuck, for their help and encouragement, and my thanks are also due to the senior technologist, Mr. A. Cooper, for his assistance in preparing the tables.

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HEREDITARY ELLIPTOCYTIC HAEMOLYTIC ANAEMIA

BY

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The erythrocytes of the camel are normally oval, whereas in man such cells are exceptional and comprise only 1 to 15% of the red cells of most normal people. A pathological increase in oval cells is found in some cases of iron deficiency and other microcytic anaemias, and there may commonly be more than 25% of oval cells among the erythrocytes in macrocytic anaemia (Florman and Wintrobe, 1938). Such symptomatic elliptocytosis (syn. ovalocytosis) is to be differentiated from hereditary elliptocytosis, which was first described by Dresbach in 1904. In the hereditary condition, rod forms are common in addition to the more oval elliptocytes, and altogether the abnormally shaped cells may constitute 50–90% of all the red cells (Wintrobe, 1956). Rod forms are not usually found among the elliptocytes of normal or anaemic blood. Hereditary elliptocytosis is transmitted as a simple Mendelian dominant, either sex being affected. It is said to occur in about 0.04% of the general American population (Wyandt, Bancroft, and Winship, 1941). Amongst affected people, the trait is said to be quite harmless in 88% of cases (Penfold and Lipscomb, 1943), but in the remainder haemolysis occurs with or without anaemia.

In the present paper an affected family is described, of which one member also suffered from haemolytic anaemia and was cured of this by splenectomy.

Case Report

A bookmaker, Homer S., aged 67 years, was admitted to hospital as an emergency on April 25, 1956. He gave a 12 hours' history of central abdominal pain radiating into the right iliac fossa, associated with nausea and anorexia, and a feeling of abdominal distension. He had had a similar, but milder, attack six months previously, which had settled on conservative treatment at home. His previous health had otherwise been good apart from an attack of typhoid fever associated with jaundice at the age of 28 years. A gangrenous appendix was removed under general anaesthesia and a drain inserted. Oxytetracycline was given intravenously for one day and orally for a further three days, until a total of 3.5 g. had been given.

On the third day after operation jaundice developed. Examination of the blood at that time showed that most of the patient's erythrocytes were oval, with some rod forms (Fig. 1). After further investigations a diagnosis was made of hereditary elliptocytosis with haemolytic anaemia associated with splenomegaly. The detailed haematological findings which went to prove this diagnosis have been listed in Table I and in the subsequent text.

After operation, intermittent pyrexia up to 100° F. lasted over six weeks. The jaundice persisted with some variation in intensity. The patient went home after two months with instructions to attend as an out-patient while investigations of haemolysis were

FIG. 1.—Peripheral blood (×750) showing elliptocytes and rod forms.

TABLE I
LABORATORY FINDINGS IN EACH MEMBER OF THE FAMILY

Name	Date	Hb (g./100 ml.)*	R.B.C. (m./c.mm.)	Ovalocytes	Rod Forms	Reticulocytes (%)	M.C.V. (c.μ.)	M.C.H.C. (%)	Excess Urinary Urobilin and Urobilinogen	Serum Bilirubin (mg./100 ml.)	Thymol Flocculation	Serum Alkaline Phosphatase (K.-A. Units)	Direct van den Bergh Reaction	Blood Group Studies
Homer S.	25/4/56													
(Appendicectomy)	28/4/56	13·8		++	+				++++	13·2	−	7	+	O, MNS, p, CDe/cde, C^w −ve, Lu (a−), Kell −ve, H +ve, Le (a+b−)
	4/5/56									18·7		16	+	
	11/5/56									4·3	−	11	+	
	25/5/56	8·3	2·5	++	+	30	87	35	++					
	2/6/56	7·4				30								
	9/6/56	10·1	2·8			12								
	18/6/56	10·8	3·2	++	+	5	91	37						
	21/7/56	11·1	3·5	++	+	7			−	3·4	−	9	±	
	23/7/56													
(Splenectomy)	24/7/56	14·8	4·5			6								
	28/7/56					2								
	31/7/56	15·5	4·5	++	+	1								
	13/8/56								−	0·6	−	10	−	
	20/8/56	15·4	5·0	++	+	< 1								
	17/6/57	16	5·5	++	+		92	38						
Jason S.	18/6/56	17·5	5·5	++	+	< 1	78	40	+	1·3	−	7	±	A_2, MMS, Pw, CDe/cde, C^w −ve, Lu (a−), Kell −ve, H +ve, Le (a+b−)
Penelope S.	28/6/56	14·8	4·8	−	−	< 1	81	38	−	0·6	−	24	−	O, MNS, p, CDe/CDe, C^w −ve, Lu (a−), Kell −ve, H +ve, Le (a−b+)
Helen S.	28/6/56	14·1	4·25	−	−	< 1	92	36	−	0·4	−	16	−	A_2, MNS, P, CDe/CDe, C^w −ve, Lu (a−), Kell −ve, Le (a−b−)
Argus S.	18/6/56	16·0	5·5	++	+	2	82	38	−	1·7	−	9	±	A_2, MNS, p, CDe/cde, C^w −ve, Lu (a−), Kell −ve, H +ve, Le (a+b−)
Diana S.	28/6/56	16·3	5·0	−	−	< 1	86	38	−	0·7	−	23	−	A, MMS, p, CDe/cDE, C^w −ve, Lu (a−), Kell −ve, H −ve, Le (a−b−)

* Hb 100% = 14·8 g. per 100 ml. blood (oxyhaemoglobin—M.R.C. photometer).

being completed. When he was readmitted for splenectomy one month later, he was still slightly jaundiced and the edge of the spleen was felt 6.5 cm. below the costal margin.

The spleen, which was surrounded by fine adhesions, was removed at operation on July 23, 1956. The gall bladder was distended and would not empty on manipulation. No gall stones were palpated and no accessory spleens were found. Fresh splenic smears showed erythrophagocytosis and an excess of granulocytes and nucleated red cells.

Appearance and Histology of the Spleen

The spleen weighed 537 g. and measured 17 × 10 × 5 cm. The cut surface resembled raw liver and the normal Malpighian pattern was indistinct. Histological examination revealed moderate congestion of the splenic pulp with marked prominence of the reticulo-endothelial cells, which lined the pulp spaces. Many instances of red cell ingestion were seen, and small haemosiderin granules abounded. There was no evidence of lymphoid hyperplasia, and the appearances were consistent with a non-neoplastic haemolytic process.

Progress After Splenectomy

Convalescence from splenectomy was rapid and uneventful. The jaundice disappeared within three days and has not since returned. The patient has had a sense of well-being such as he has not felt for a long time. Haematological investigations made five months after splenectomy showed a persistence of the characteristic oval and rod-shaped cells, but a complete absence of abnormal haemolysis. Details of these studies are included in Table I and in the text.

Red Cell Survival Studies Using ^{51}Cr

Survival of red cells was studied in May, 1956, two months before splenectomy, and again in November, 1956, five months after operation. The same technique was used on each occasion.

$Na_2{}^{51}CrO_4$, with an initial specific activity of 25 μc./μg., was obtained in solution in isotonic saline from the Radiochemical Centre, Amersham, England. Fifteen millilitres of the patient's blood were added to 50 μc. ^{51}Cr and 500 units of heparin in a sterile glass container and the mixture was allowed to stand at room temperature for one hour. The red cells were then washed twice with cold, sterile, isotonic

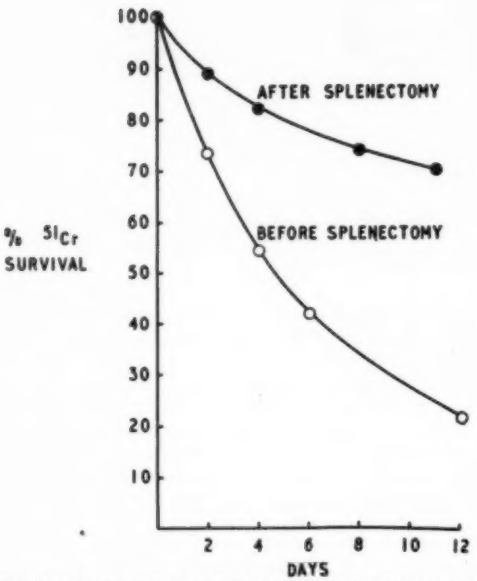

FIG. 2.—Survival of patient's own red cells labelled with ^{51}Cr before and after splenectomy.

saline, resuspended in saline, and 10 ml. of this suspension was injected intravenously. Standard dilutions were made in duplicate from what remained of the suspension; a dilution was also prepared from the supernatant fluid obtained by centrifugation of the cell suspension.

One blood sample was taken from the opposite arm 30 minutes after the injection, and four further blood samples were taken at intervals of two to six days. The haematocrits of all blood samples and of the labelled red cell suspension were measured in duplicate in Wintrobe tubes centrifuged at 3,000 r.p.m., at a radius of 15 cm. for 55 minutes. All readings were corrected for trapped plasma using the data of Chaplin and Mollison (1952). After removing enough blood for haematocrit measurement, the remainder of each sample was haemolysed with saponin.

The radioactivity of all haemolysed blood samples and standard dilutions was measured in a scintillation counter ("Ekco," type N550), 6,000 counts being obtained in all cases. Correction was made for radioactive decay and for variation in the characteristics of the counting apparatus by reference to a standard dilution of ^{51}Cr. The radioactivity of blood samples was expressed as counts per minute per millilitre of red cells, by dividing the counts per minute for whole blood by the corrected haematocrit. These values were then expressed as percentages of the radioactivity of the initial sample withdrawn 30 minutes after the injection of labelled cells.

In the study carried out before splenectomy, the total red cell volume was finally measured on the twelfth day by the injection of the patient's red cells labelled with ^{32}P, the blood samples and standard dilutions being counted on a G.M. liquid counter (Veall, 1948). Total red cell volume measurements at the beginning and end of the study before splenectomy, and at the beginning of the subsequent study, are shown in Table II; in the second study the red cell volume was assumed to be constant during the period of observation and was determined only once.

TABLE II

TOTAL RED CELL VOLUME AND HALF-TIME OF SURVIVAL OF ^{51}Cr BEFORE AND AFTER SPLENECTOMY

	Total Red Cell Volume (litres)	Half-time of ^{51}Cr Survival (days)
Before splenectomy	Initial, 1·09 Final, 1·13	4·5
After ,, ...	1·64	24

The ^{51}Cr survival curves before and after splenectomy are shown in Fig. 2. The half-time of ^{51}Cr survival (Table II) before splenectomy was reduced to 4.5 days, whereas extrapolation of the curve after splenectomy showed a half-time of survival of 24 days, which is within the normal range (Read, Wilson, and Gardner, 1954; Mollison and Veall, 1955). No correction has been made for total red cell changes during the first study. The observed change in total red cell volume is slight and of doubtful significance, particularly as the second measurement utilized ^{32}P-labelled red cells, a method known to give occasional over-estimates of red cell volume (Mollison and Veall, 1955).

From the observed ^{51}Cr survival before splenectomy, the "true survival" of red cells was calculated as suggested by Jones and Mollison (1956), using the data for normal ^{51}Cr survival of Mollison and Veall (1955). The true survival was then plotted (Fig. 3) and the tangent to the initial slope extrapolated to the time axis to give an estimate of mean red cell life (Dornhorst, 1951). Before splenectomy, the mean cell life was nine days. After splenectomy, the normal half-time of ^{51}Cr survival of 24 days can be taken to indicate a normal red cell life of about 110 days (Mollison and Veall, 1955; Jones and Mollison, 1956).

Other Investigations Made before Splenectomy

The saline osmotic fragility of the red cells (Dacie, 1956a) was slightly increased (control 0.44–0.34% saline, patient 0.56–0.40% saline). Incubation of such suspensions at 37° C. for 24 hours (Dacie, 1956b) accentuated their difference from the control. Autohaemolysis tests, where whole blood is incubated at 37° C. for 24 and 48 hours (Dacie, 1956c), and the mechanical red cell fragility (Dacie, 1956d) were within the normal range. Bone marrow examination showed an active normoblastic reaction, but no normoblasts were elliptical. Reticulocytes were mostly round, so that the elliptocytes were fully apparent

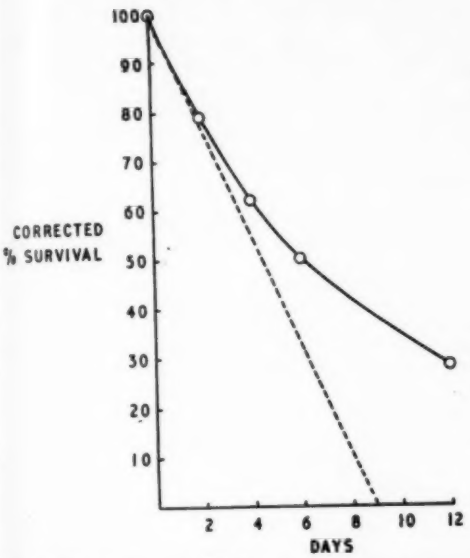

FIG. 3.—The "true survival" of red cells before splenectomy.

only at the erythrocyte level of maturity. Examination of wet sealed films confirmed the elliptocytosis and did not show true spherocytes. The serial dilution direct antiglobulin test was negative: no auto- or pan-haemagglutinins or haemolysins were detected in saline or in albumin at 4° C., 18° C., and 37° C.: the Donath-Landsteiner test was negative: the acid-serum haemolysis test was negative: no immune Rhesus or Kell antibodies were detected in saline or albumin or by using the indirect antiglobulin technique. There was no evidence of auto-immunization. An intravenous cholecystogram outlined the common bile duct, but the gall-bladder was not seen. No calculi were seen.

After splenectomy the haemoglobin was examined electrophoretically and did not show the presence of any abnormal haemoglobin. The saline osmotic fragility of the red cells was estimated again and was still slightly increased (control 0.48–0.34% saline, patient 0.54–0.38% saline).

Family Studies

Five direct descendants of the patient (Homer S.) were studied. His two sons, Jason, aged 41 years, and Argus, aged 39 years, both showed the same elliptocytosis as their father, but they supplied no more than suggestive evidence of subclinical haemolysis (Table I). Neither son had felt in any way unwell and there had been no clinical jaundice at any time, nor was there detectable splenomegaly. The saline osmotic fragilities of their peripheral red cells were normal.

The two daughters of Jason, Penelope, aged 10 years, and Helen, aged 7 years, were studied at the same time as their father. They showed no elliptocytosis and appeared normal in all respects apart from a serum alkaline phosphatase activity in Penelope of 24 King-Armstrong units of doubtful interpretation (Table I). There was no history of jaundice.

Diana, aged 9 years, the daughter of Argus, also showed no elliptocytosis and was well; she had a serum alkaline phosphatase activity of 23 King-Armstrong units. There was no history of jaundice.

Discussion

The factors which determine the unusual shape of the erythrocytes in hereditary elliptocytosis are unknown. There are apparently no abnormalities in the haemoglobin as determined by the alkaline denaturation test and by haemoglobin electrophoresis (Letman, 1955), but Selwyn (1953) has shown that glucose reduces the amount of erythrocyte autohaemolysis *in vitro* to a lesser degree than in the normal. The mean corpuscular volume and the mean corpuscular haemoglobin concentration of elliptocytes have usually been reported as normal. This is also the case in the three affected members of this family.

Haemolytic anaemia with elliptocytosis has been described where the latter trait was present in both parents (Wyandt *et al.*, 1941). The frequency of such a union must be very rare. Where the inheritance of the elliptocytosis comes from only one parent, most patients remain free from apparent haemolysis, but some develop a haemolytic anaemia (Motulsky, Singer, Crosby, and Smith, 1954; Lipton, 1955). It is not possible to investigate the antecedent heredity in our patient, both his parents being dead.

Goodall, Hendry, Lawler, and Stephen (1953) have found evidence for linkage of the gene for elliptocytosis to the cde (Rhesus blood group) combination of antigens. Our findings lend support to this work because the cde combination is present in the propositor and his two sons, who all have elliptocytosis, and not in the three granddaughters, who have normal peripheral blood.

Our studies support the view that the nucleated precursors of elliptocytes are round, the anomaly first making its appearance in the reticulocyte stage (Schartum-Hansen, 1935). Letman (1955) noted an increase in saline osmotic fragility following splenectomy in a case of hereditary elliptocytosis with haemolytic anaemia. Here the fragility was slightly abnormal before splenectomy, but was not increased by splenectomy. The saline osmotic fragility of the elliptocytes of the two sons appeared to be within normal limits.

It is quite possible that subclinical haemolysis had been present for some time, perhaps years, before the patient was ever seen in hospital, but it seems probable on clinical evidence that haemolysis was at least greatly increased, even if not initiated, by some factor operating at the time of his admission for appendicectomy. The rate of haemolysis appeared to have diminished three months later. Jaundice was then still apparent clinically, but disappeared immediately after splenectomy.

There are certain features concerning the two sons which are suggestive of slightly abnormal haemolysis. Both have a raised serum bilirubin level, a positive direct van den Bergh test, and one has a slight excess of urobilinogen in the urine. The presence of abnormal haemolysis could be proved only by differential cell agglutination survival studies introduced by Ashby (1919) or by radioactive labelling of red cells.

Several studies have been made of red cell survival in hereditary elliptocytosis without anaemia (Trinick, 1948; Berlin and Hedenstedt, 1952; Motulsky *et al.*, 1954). Most recent studies have shown normal survival of the elliptocytes when transfused into normal subjects. One patient, reported by Motulsky and his associates (1954), had a shortened red cell survival time of 45 days and had also other evidence of haemolysis, but was not anaemic.

Information about red cell survival in hereditary elliptocytic haemolytic anaemia is scanty. Splenectomy has been reported to be of benefit in several cases (Wilson and Long, 1953; Dacie, Mollison, Richardson, Selwyn, and Shapiro, 1953). In the present case the shortened mean cell survival time had returned to normal five months after operation, and splenectomy was clearly of benefit.

Summary

A case is described of hereditary elliptocytosis with an associated haemolytic anaemia which responded well to splenectomy. The degree of haemolysis had increased at the time of surgery for acute appendicitis. The red cell survival time using ^{51}Cr was short before splenectomy, but normal after operation. The saline osmotic fragility was slightly increased, but was not affected by splenectomy. No obvious change was noted in the appearance of the elliptocytes after operation.

The two sons of the patient have elliptocytosis without anaemia. The father and sons all have the cde combination of antigens, which is absent in three granddaughters, who show no elliptocytosis.

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AN EVALUATION OF A NEW DIAGNOSTIC TEST FOR SYSTEMIC LUPUS ERYTHEMATOSUS

BY

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In a disease with such widespread and variable manifestations as systemic lupus erythematosus the diagnosis, at least in the early stages, often depends upon a clinical suspicion supported by laboratory investigations, notably the demonstration of lupus erythematosus (L.E.) cells. While this test is highly specific for systemic lupus erythematosus it has these disadvantages. In a few patients L.E. cells may not be demonstrable until late in the course of the illness and in a very few cases they may perhaps be absent throughout. The test requires expert interpretation if it is to be reliable.

A really simple screening test that could be applied to the many patients with apparent rheumatoid arthritis, cutaneous lupus erythematosus, rheumatic fever, and other diseases which may be mimicked by systemic lupus erythematosus, would be invaluable for the early diagnosis of this more serious illness.

Recently Jones and Thompson (1957) have described a simple protein precipitation test which they have found useful in the diagnosis of systemic lupus erythematosus and which apparently gives false positive results only in liver diseases and myelomatosis, conditions which do not usually cause clinical confusion with systemic lupus erythematosus.

The present report summarizes experience of this test applied on 1,042 occasions to serum from more than 800 patients with various diseases, including systemic lupus erythematosus and discoid lupus erythematosus. Attempts have also been made to identify the protein fraction precipitated in the test.

Methods

The test reagent consists of a 12% solution of paratoluene sulphonic acid in glacial acetic acid. This should give a clear solution which keeps indefinitely. Old discoloured preparations of paratoluene sul-

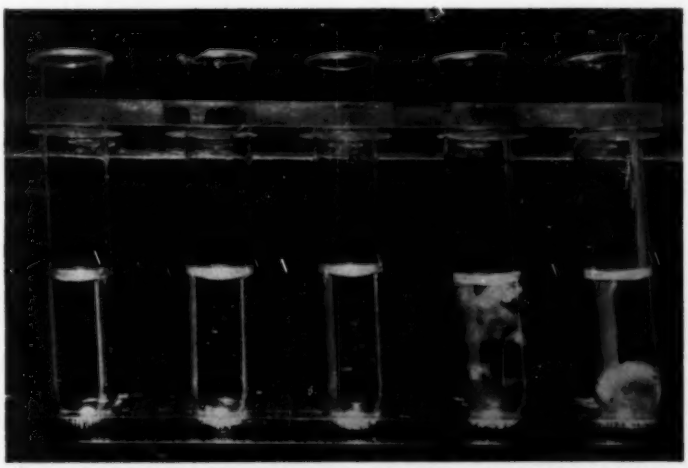

FIG. 1.—Grading of results of paratoluene sulphonic acid tests. Reading from left to right, the above tubes were read as 0, ±, +, ++, +++.

phonic acid were found to be unsuitable and caused a high proportion of false positive results. One millilitre of the reagent was pipetted into a small test-tube and to this was added 0.1 ml. of serum. In a positive test a precipitate appears at once, but in this series the tubes were left for 20 to 30 minutes, then vigorously shaken and examined for precipitation with indirect lighting against a dark matt background. The degree of precipitation was recorded as shown in Fig. 1.

With plasma instead of serum, precipitation occurred more frequently and rendered the test rather non-specific.

Interpretation

It soon became obvious that minor degrees of precipitation (either ± or +) occurred not infrequently with sera from patients with many diseases. On the other hand most cases of active systemic lupus erythematosus gave ++ or +++ precipitation. This was called "significant precipitation."

Results

Systemic Lupus Erythematosus.—This group consists of 25 patients who had a clinical picture compatible with the diagnosis of systemic lupus erythematosus and all of whom had shown a positive L.E. cell test at some stage in the illness. Table I shows the results of paratoluene sulphonic acid tests, the intensity of L.E. tests on the same blood samples and the activity of the disease at the time of testing. The last assessment was based on the patients' clinical condition, temperature, and erythrocyte sedimentation rate.

Of 14 patients with active untreated disease, the test gave ++ or +++ precipitation in 11. With one exception (K. O.) there was fair correlation between the amount of precipitate and the activity of the disease. In two of the patients repeated tests were done before and during treatment and the amount of precipitate found did correspond fairly well with the activity of the disease (Fig. 2).

One patient (P. R.), in whom the disease was in remission and who was no longer on treatment, showed no L.E. cells at the time of testing and the paratoluene sulphonic acid test was negative.

From 10 patients tested for the first time while on steroid therapy, only two sera showed significant precipitation. In these patients the correlation between the amount of precipitate and the disease activity was poor.

Probable Systemic Lupus Erythematosus.—Three patients with a very suggestive clinical picture failed to show L.E. cells despite repeated tests. None of these three showed significant precipitation with the paratoluene sulphonic acid test, but two gave a + reaction (Table II).

TABLE I

RESULTS OF PARATOLUENE SULPHONIC ACID TESTS ON 25 PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS CORRELATED WITH THE THERAPY, DISEASE ACTIVITY, AND L.E. TEST AT TIME OF TESTING

Patient	Therapy	Disease Activity*	L.E. Test	Paratoluene Sulphonic Acid Test
E. S. ..	Nil	+++	+++	+++
Y. S. ..	,,	+++	+++	+++
G. G. ..	,,	+++	+++	+++
G. Gr. ..	,,	+++	++	++
A. H. ..	,,	+++	+++	++
C. M. ..	,,	+++	++	++
K. O. ..	,,	+++	+	0
P. S. ..	,,	++	+	+++
E. B. ..	,,	++	++	+++
E. S. ..	,,	++	++	++
K. M. ..	,,	++	++	++
L. L. ..	,,	+	+	++
M. Q. ..	,,	+	+	+
S. M. ..	,,	+	+	±
P. R. ..	,,	0	0	0
E. O. ..	Prednisone	+++	++	0
P. Gr. ..	Prednisolone	+++	+	0
L. B. ..	Prednisone	+++	++	0
J. W. ..	Cortisone	++	0	+++
Q. J. ..	,,	+	0	+
B. W. ..	Prednisolone	+	0	0
B. B. ..	,,	+	0	0
J. W. ..	Prednisone	+	0	+
F. T. ..	Prednisolone	+	+	0
Ph. L. ..	,,	+	0	++

* The disease activity and intensity of the L.E. test are graded from 0 to +++.

TABLE II

RESULTS OF PARATOLUENE SULPHONIC ACID TESTS ON 812 PATIENTS WITH CONDITIONS OTHER THAN PROVED SYSTEMIC LUPUS ERYTHEMATOSUS

Disease	No. of Patients Tested	Paratoluene Sulphonic Acid Test				
		0	±	+	++	+++
?S.L.E.	3	1	—	2	—	—
Discoid L.E. ..	51	44	1	6	—	—
Others	758	596	61	59	28	14
Total ..	812	641	62	67	28	14

Discoid Lupus Erythematosus.—All the 51 patients in this group had been diagnosed by an experienced dermatologist. Eight showed minor evidence of some systemic upset such as Raynaud's phenomenon, arthralgia, lymphadenopathy, an accelerated blood sedimentation rate, leucopenia, or serum protein abnormalities. All had negative L.E. tests on one or more occasions. None of the 51 sera from these patients showed significant precipitation, but seven showed a light precipitate (either ± or +, Table II).

Other Diseases and Normal Subjects.—Serum from 758 other subjects was tested. This included 38 healthy blood donors, 36 normal pregnant women, and 684 ward or clinic patients. The diagnosis in these cases was obtained from the

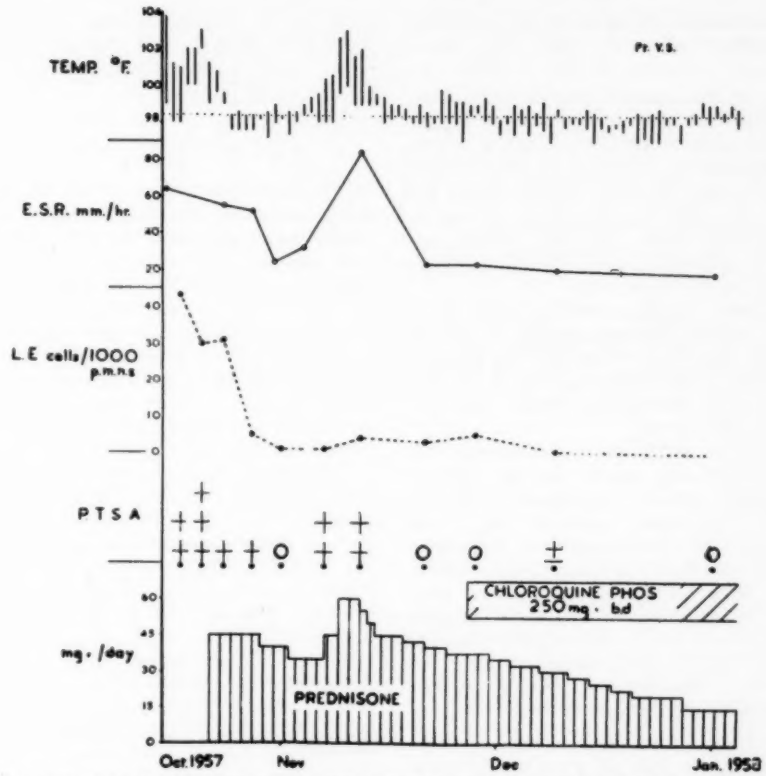

FIG. 2.—Effect of steroid treatment on paratoluene sulphonic acid test result. The test results are graded as in Fig. 1, and are shown in the chart as a dot with the result read vertically above.

hospital case notes or in some cases from personal examinations. Many patients had multiple tests.

Serum from 42 of these patients gave significant precipitation (Table III), but minor degrees of precipitation were encountered in no less than 129 others. The remaining patients gave negative test results.

Almost half of the positive tests in this group were associated with obstructive or hepatocellular jaundice and the remainder included a high proportion with diseases of the reticulo-endothelial system, others with syphilis, rheumatoid arthritis, and in one case with ankylosing spondylitis with peripheral joint involvement. In view of the alleged occurrence of L.E. cells in drug reactions, it is of interest that the only patient with a drug reaction in this series did have a positive paratoluene sulphonic acid test. The L.E. test on this patient was negative.

TABLE III

PATIENTS WITH DISEASES OTHER THAN SYSTEMIC LUPUS ERYTHEMATOSUS SHOWING SIGNIFICANT (++ OR +++) PRECIPITATION WITH PARATOLUENE SULPHONIC ACID REAGENT

Diagnosis	No. of Patients with Positive Test	No. of Patients Tested
Cirrhosis	11	36
Infective hepatitis	2	4
Obstructive jaundice	6	11
Rheumatoid arthritis	4	46
Ankylosing spondylitis	1	8
Myelomatosis	2	3
Sarcoidosis	1	2
Lymphoma or leukaemia	2	7
Late syphilis	3	20
Penicillin sensitivity	1	1
Miscellaneous conditions	9	620
Total	42	758

In none of these 42 patients with positive tests was the clinical picture suggestive of systemic lupus erythematosus and in 30 cases L.E. tests

on the same samples of blood as were used for the paratoluene sulphonic acid tests proved negative.

Nature of the Precipitate.—The precipitates produced from nine positive sera (four from patients with systemic lupus erythematosus and five from patients with other conditions) were washed twice with distilled water, smeared on filter paper and dried. In every case the precipitate stained intensely with light green dye and weakly with Sudan black, indicating a considerable protein content with a little lipoprotein.

The precipitate was invariably insoluble in distilled water but moderately soluble in normal sodium hydroxide. Unfortunately, the resulting solution, when neutralized, showed no electrophoretic mobility, presumably because the protein had been denatured.

Scrutiny of the serum protein electrophoretic patterns, however, did suggest that the patients with a strongly positive paratoluene sulphonic acid test had high serum γ globulin levels. The absolute amount of serum γ globulin was therefore estimated on 24 sera and correlated with the paratoluene sulphonic acid result. The γ globulin was estimated from the total serum proteins as measured by the biuret method, and from the scanning of the paper electrophoretic pattern.

It can be seen from Table IV that, while there was considerable overlap between groups, the

TABLE IV

SERUM γ GLOBULIN VALUES CORRELATED WITH RESULTS OF PARATOLUENE SULPHONIC ACID TESTS ON THE SAME SERA

Paratoluene Sulphonic Acid Test Result	0	± or +	++	+++
No. of patients tested	5	6	7	6
Mean serum γ globulin (g.%)* ..	1·36	1·81	2·2	2·8
Range of serum γ globulin (g.%) ..	1·2–1·6	1·1–2·7	1·3–2·5	1·4–4·0

* Analysis of variance on the original γ globulin values showed a difference between groups significant at the 5% level.

mean serum γ globulin of each group did increase consistently with the intensity of precipitation produced by paratoluene sulphonic acid, suggesting that the latter may be precipitating γ globulin.

This was confirmed on 25 ml. of pooled serum from two patients with systemic lupus erythematosus by using ether fractionation to separate various protein fractions.† The freeze-dried protein fractions along with a freeze-dried sample of the original serum were then reconstituted in half the

† This was done by Dr. R. A. Kekwick at the Lister Institute of Preventive Medicine, using the technique of Kekwick and MacKay (1954).

TABLE V

RESULTS OF PARATOLUENE SULPHONIC ACID TESTS ON PROTEIN FRACTIONS OF POOLED SYSTEMIC LUPUS ERYTHEMATOSUS SERUM

Pooled whole serum	++
Albumin	0
α and β lipoprotein	±
α and β lipoprotein minus unstable lipoid	0
α_2 and β globulins	0
γ globulin	++

theoretical volume of distilled water to allow for wastage, and tested with the paratoluene sulphonic acid reagent (Table V). Only the whole serum and the γ globulin fraction gave positive results. The latter fraction was checked by paper electrophoresis and found to consist almost wholly of γ globulin with a mere trace of β globulin. This fraction contained no lipoprotein, so it seems likely that the trace of lipoprotein found in the analysis of precipitate from whole serum had been adsorbed during precipitation.

A solution of γ globulin prepared by ether fractionation of pooled normal sera (obtained from the Lister Institute of Preventive Medicine) gave a positive paratoluene sulphonic acid test, the intensity being proportional to the concentration of γ globulin. Significant precipitation occurred with solutions containing 4% or more of γ globulin.

Since the paratoluene sulphonic acid reagent precipitates γ globulin from sera of normal people and of patients with various diseases, it seems likely that the test is related to an increased quantity of γ globulin or some fraction thereof, rather than to an abnormal γ globulin fraction.

Discussion

It is obvious that the paratoluene sulphonic acid test cannot be used alone to support a diagnosis of systemic lupus erythematosus and that the more specific L.E. cells must be sought in all suspected cases. Even as a screening test the paratoluene sulphonic acid precipitation is not ideal because of the occasional negative results in florid untreated systemic lupus erythematosus. It is of interest that the one striking example of this (patient K. O. in Table I) had a severe nephrotic syndrome with massive proteinuria and low total serum proteins with a serum γ globulin of only 1.2 g. per 100 ml.

However, because of its simplicity, the test may prove useful provided that a positive result is interpreted as indicating a raised serum γ globulin component which may be due to systemic lupus erythematosus as well as other diseases.

Although in untreated cases of systemic lupus erythematosus the paratoluene sulphonic acid test

does seem to parallel the severity of the illness, this association is not always present after steroid therapy. The erythrocyte sedimentation rate will then prove a more reliable guide to the activity and progress of the disease.

Summary

A simple serum protein precipitation test, using paratoluene sulphonic acid in glacial acetic acid, has been examined for its value in the diagnosis of systemic lupus erythematosus. A positive test was found to depend upon an excess of serum γ globulin.

Thus it was positive in the majority of cases of untreated active systemic lupus erythematosus and negative in all cases of discoid lupus erythematosus. It was positive, however, in a number of conditions other than systemic lupus erythematosus which were associated with a raised serum γ globulin level.

With the exception of rheumatoid arthritis, these other conditions were generally readily distinguishable from systemic lupus erythematosus on clinical grounds.

We wish to thank Dr. A. B. Anderson for providing facilities for much of this work and Dr. R. A. Kekwick for his help with the protein fractionation.

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THE DIFFERENTIATION OF MYELOMATOSIS FROM OTHER CAUSES OF BONE MARROW PLASMACYTOSIS

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The proportion of plasma cells in aspirates of bone marrow is occasionally increased in conditions other than myelomatosis (Bing, 1940; Bayrd, 1948; Fadem and McBirnie, 1950; Fadem, 1952; Bessis, 1956). In cases of this sort (the so-called "plasmacytic response"), one may see immature cells, sometimes in clumps, cytoplasmic vacuolation, multinucleated cells, and degenerative changes of different kinds (Hayhoe and Smith, 1951). Paris and Bakke (1956) reported a case of agranulocytosis in which the marrow aspirate contained 60.2% of plasma cells. They observed that the occasional difficulty in distinguishing such plasmacytoses from myelomatosis is analogous to the difficulty in distinguishing a leukaemoid reaction from leukaemia. If, as may happen, myelomatosis cannot be excluded on clinical or other grounds a diagnostic impasse may result.

Fadem (1952) compared the marrow findings in 110 cases of plasmacytic response with those in 52 cases of myelomatosis and was able to establish a number of broad differences between the two groups. For example, the marrow in plasmacytic response was usually normally cellular, and the plasma cells were mainly mature, uniformly distributed, often related to reticulum cells, and rarely more numerous than 20% of the total count. In myelomatosis, on the other hand, the marrow was most often hypocellular, and the plasma cells mainly abnormal, shifted to the left, focal in distribution, rarely associated with reticulum cells, and usually more numerous than 20%.

It is possible to criticize such differential criteria in that they are general statistical trends which are true of large groups but afford only a low degree of probability in individual cases. Thus Paris and Bakke (1956) rely particularly on the higher proportion of proplasmacytes in myelomatosis, but they consider that borderline cases may occur in which doubt may remain as to the true nature of the process. A narrower and more objective criterion would, therefore, seem desirable.

MacCarty (1929, 1936) called attention to the enlarged nucleolus of the cancer cell and suggested its diagnostic value. Quensel (1928a, b, and c) examined the cells of pleural and peritoneal effusions using a supravital technique in which nucleoli were stained by methylene blue. The atypical cells of malignant effusions frequently showed nucleolus/nucleus ratios in the upper part of the range 0.20 to 0.66. The normal endothelial cells of the same fluids showed nucleolus/nucleus ratios of 0.12 to 0.20.

The nucleolus of the myeloma cell is always abnormal and often strikingly hyperplastic (Bessis, 1956). Its importance in diagnosis has been stressed by Streicher, Sandkühler, Roth, and Schwenkenbecher (1953). The mere observation of enlarged nucleoli may not always be sufficient for diagnosis, however, since plasma cells which are responding to antigenic stimulation may also show this feature. It will be suggested in this paper that assessment of the nucleolus/nucleus (n/N) ratio gives a more reliable morphological distinction between plasmacytic responses and myelomatosis.

Materials and Methods

Smears of marrow obtained by aspiration biopsy from 10 consecutive cases of myelomatosis were compared with those from 10 consecutive assorted cases in which the proportion of plasma cells was increased. The normal marrow plasma cell percentage was discussed by Hayhoe and Smith (1951), who concluded that 2% and more were pathological: their conclusions are adopted here. The relevant details of these cases are set out in the Tables. Nucleoli were demonstrated negatively by applying the Feulgen reaction for desoxyribonucleic acid as follows (Gardikas and Israels, 1948, though using routine methyl alcohol fixation): (1) Washed in tap-water for 10 minutes; (2) washed in distilled water for two minutes; (3) placed in N HCl (room temperature) for two minutes, then (4) in N HCl at 60° C. for eight to 10 minutes, then (5) rinsed in N HCl (room temperature); (6) rinsed in distilled water; (7) stained with leucobasic fuchsin (Schiff reagent) for one and

TABLE I
RELEVANT FEATURES OF 10 CASES OF MYELOMATOSIS

Case	Marrow Myeloma Cells (%)	Nucleolus/ Nucleus Ratio	Remarks
1	39	0·49	Multiple focal osteolytic deposits
2	11	0·47	" " " "
3	60	0·46	Plasma cell leukaemia
4	12	0·43	Multiple focal osteolytic deposits
5	100	0·42	Severe osteoporosis; aspirate probably from focal deposit
6	23	0·40	Plasmacytoma of humerus; slow generalization of disease over following 3 years
7	37	0·39	Diffuse marrow involvement; presented as "primary" amyloidosis
8	21	0·39	Severe osteoporosis
9	50	0·34	Diffuse marrow involvement
10	11	0·32	Multiple focal osteolytic deposits; extensive amyloidosis

TABLE II
RELEVANT FEATURES OF 10 CASES OF PLASMACYTIC RESPONSE

Case	Final Diagnosis	Marrow Plasma Cells (%)	Nucleolus/ Nucleus Ratio	Remarks
1	Adenocarcinoma of colon	7·0	0·29	E.S.R. 137 mm./hr. Atypical plasma cells suspicious of myeloma. Plasma proteins, total 8·8 g., globulin 5·7 g.
2	Tabes dorsalis	13·0	0·29	Many atypical plasma cells, suspicious of myeloma
3	Carcinomatosis	6·0	0·27	
4	Aplastic anaemia	15·0	0·27	
5	Pulmonary tuberculosis	5·0	0·26	
6	Congenital syphilis; probable rheumatoid arthritis	3·6	0·25	E.S.R. 130 mm./hr. plasma proteins, total 7·3 g., globulin 4·9 g.
7	Cirrhosis of the liver	4·0	0·25	
8	Subacute nephritis; chronic rheumatic endocarditis	9·0	0·24	Many atypical plasma cells, suspicious of myeloma. Plasma proteins, total 7·1 g., globulin 4·2 g.
9	Aplastic anaemia	13·0	0·24	
10	Frontal astrocytoma; chronic liver disease	3·4	0·22	E.S.R. 120 mm./hr.

a half to two hours; (8) put through two or three changes of SO_2 water, each of one minute's duration; (9) washed in tap-water for 10 to 15 minutes; (10) dehydrated in graded alcohols, cleared in xylol, and mounted.

The SO_2 water was prepared from sodium metabisulphite by treatment with hydrochloric acid (5 ml. 10% $Na_2S_2O_5$, 5 ml. N HCl, water to 100 ml.).

A simpler modification of this method omitting stages 1, 3, and 5 was found quite satisfactory. Careful control of time and temperature during the period of hydrolysis (stage 4) is important in securing consistent results. Counterstaining is not essential but facilitates the recognition of plasma cells by showing their cytoplasm. To this end the smears may be immersed in 0.01% fast green FCF in 95% alcohol (Lillie, 1954) for a few seconds, or in Jenner's stain (Gardikas and Israels, 1948) for 10 minutes. Counterstaining follows stage 9 of the technique.

Nuclear and nucleolar diameters were measured with a Zeiss screw micrometer eyepiece mounted on a monocular microscope. All measurements were made in a darkened room, using the same instrument throughout. In order to minimize factors of variation such as thickness of smear, intensity of staining, or irregularity of nuclear and nucleolar outline, 25 cells were measured in each case. These were sought in as many random areas as were necessary. An average of the 25 n/N ratios was then taken. Degenerate cells and those with obscured nucleoli were ignored. Reticulum cells and the earlier normoblasts occasionally simulated the chromatin pattern of plasma cells; where doubt could not be resolved the cell in question was rejected. Many normal mature plasma cells were bypassed in cases of plasmacytic response because no clear nucleolus was discernible. Such cells were very rarely seen in cases of myelomatosis.

As the method of assessment was quantitative and objective, tests of reproducibility were not considered essential. A rough index of the attainable accuracy is given by the fact that in four cases (two from each group) remeasurement after a lapse of time yielded ratios agreeing with the originals to within 0.02.

Results

The average n/N ratios, calculated from the ratios of 25 random cells in each of 10 cases of plasmacytic response, ranged from 0.29 to 0.22 with a mean of 0.26. Similarly calculated in 10 cases of myelomatosis it ranged from 0.49 to 0.32, with a mean of 0.41. The boundary between the two categories is about 0.31 and there is no overlap. As nuclei and their nucleoli varied somewhat in size, especially in myelomatosis, it was considered that absolute measurements would vary too much to be useful and so were not calculated.

Though morphology was of secondary interest, certain features came to be recognized as typical of the myeloma cell nucleus. The nucleolus was usually central or slightly eccentric and there was a notably thick nucleolar membrane (Fig. 1). The nucleolar substance occasionally showed a curious bluish refractility. There was prominent nucleolus-associated chromatin in most instances. The specificity of the Feulgen reaction for D.N.A. enabled almost quantitative comparisons to be made between these nuclei and those of normal immature plasma cells in which the nucleolus, even when large, was always delicately bounded (Fig. 2). It also enabled an estimate to be made of the degree of maturity of individual cells. Normal

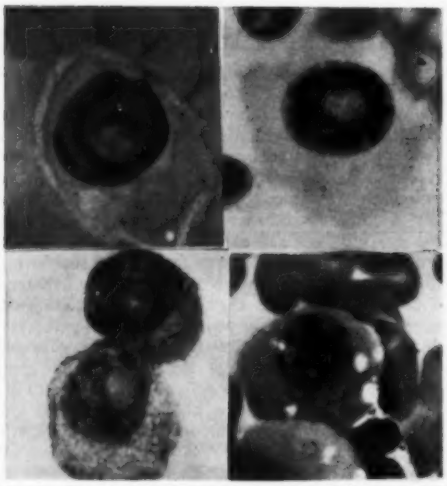

FIG. 1.—Typical myeloma plasma cells. Feulgen–Jenner, × 800.

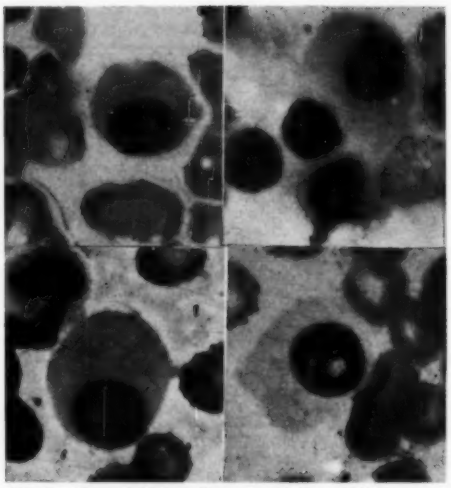

FIG. 2.—Plasma cells from four cases of plasmacytic response. Feulgen–Jenner, × 800.

mature Marschalko plasma cells were commonly seen in cases of plasmacytic response, but rarely, and doubtfully, in myelomatosis.

The Feulgen technique used (essentially that proposed by Gardikas and Israels (1948) for other haematological purposes) was found to be simpler, more delicate, and more controllable than the Unna–Pappenheim procedure. Romanowsky stains are unsuitable as they frequently obscure nucleoli completely.

Comment

The present study employs a rather small series of cases from which dogmatic conclusions may not be drawn. The results obtained by micrometry do, nevertheless, amply confirm the visual impression of greater nucleolar enlargement in myelomatosis than in other forms of marrow plasmacytosis. They suggest, moreover, that this difference in size, supplemented perhaps by differences in structure, may be of practical value in diagnosis.

There is a miscellany of diseases in which the proportion of marrow plasma cells is increased. In many of them an elevation of plasma globulins is found as well, possibly due to autoantigenic stimulation (Hayhoe and Smith, 1951). A clinical suspicion of myelomatosis may be raised, for example, by carcinomatosis, particularly when there are osteolytic secondary deposits (Marchal and Mallet, 1948, quoted by Bessis, 1956), by tuberculosis in some of its manifestations, by rheumatoid arthritis (Hayhoe and Smith, 1951), by anarthritic rheumatism (Bagratuni, 1956), by hypersensitivity states (Paris and Bakke, 1956), or by syphilis. The responsibility for diagnosis may then be felt to lie with the clinical haematologist. Case 1 of the plasmacytic response group illustrates this situation.

C. E. (Reg. No. 224913), a man aged 71, presented with a history of pain in the lower lumbar region, left loin, and left leg; he was anaemic (Hb 66%, 9.8 g.); his E.S.R. was 137 mm./hr.; his plasma proteins were raised to 8.8 g./100 ml., of which 5.7 g. were globulins; a marrow aspirate contained 7% of plasma cells, many of which were atypical. Post-mortem examination revealed an advanced adenocarcinoma of the sigmoid colon which was infiltrating the tissues of the left side of the pelvis. There was no evidence of myelomatosis.

In cases such as this, when even the marrow smears are equivocal, assessment of the n/N ratio may give a valuable clue to the correct diagnosis.

Summary

The ratio of nucleolus to nucleus in the plasma cells of 10 cases of myelomatosis was consistently greater than that in plasma cells of 10 cases of "reactive" bone marrow plasmacytosis (so-called plasmacytic response). It is suggested that this finding may be of practical diagnostic value in equivocal cases.

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THE NON-PROTEIN CONSTITUENTS OF THE PLASMA IN MYELOMATOSIS

BY

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Myelomatosis, a disease associated primarily with a defect in protein metabolism, may on occasion produce other bizarre and even misleading changes in the plasma constituents.

Isolated reports of unusual cases, such as those of Cantarow in 1935 and Nassim and Crawford in 1950, have appeared from time to time in the literature; but more comprehensive reviews show such a divergence of opinion on the frequency with which these abnormal "ancillary" biochemical findings occur that it seemed of practical value to give in condensed form the analysis on 74 proved cases of myelomatosis examined and followed in this department in the course of a much wider study of the causes and significance of hyperglobulinaemia.

The histograms refer to the levels found in the first samples submitted to the laboratory from each patient. To have grouped all the analyses carried out would, we believe, have given an artificial picture, for it is natural that unusual values have been repeated more often than normal values; moreover, "terminal" values, except in the rare instances when patients were admitted undiagnosed *in extremis*, are not of great practical value. Of greater interest are the bizarre findings encountered when the patient first comes to the doctor.

Methods

Total Protein.—This was estimated as total nitrogen by the micro-Kjeldahl distillation technique of Pregl, and, after subtraction of the non-protein nitrogen, converted to protein using a factor of 6.25.

Urea.—Urea was estimated by a modification (Harrison, 1947) of the urease-Nesslerization method of Archer and Robb (1925).

Uric Acid.—The estimation was carried out by the method of Brown (1945), which gives values not more than 0.2 mg. above the true uric acid levels except in renal damage.

Cholesterol.—Cholesterol was estimated using the Liebermann-Burchardt reaction by a modification of the methods of Bloor (1928) or Sackett (1925). The estimation was carried out on 1 ml. of serum.

Alkaline Phosphatase.—This was estimated by determining the liberated phenol from diphenyl phosphate with Folin and Ciocalteau's phenol reagent, using the micro-method of King and Armstrong (King, 1956).

One Bodansky unit is approximately equal to 2½ King–Armstrong units.

Calcium.—Calcium was estimated by direct precipitation from serum as calcium oxalate, and titration of the washed precipitate dissolved in acid, with standard permanganate solution by the method of Kramer and Tisdall as modified by Clark and Collip (1925).

Inorganic Phosphorus.—This was estimated by the method of Fiske and Subbarow (1925) using 1.2.4. aminonaphtholsulphonic acid as the reducing agent.

Results

The total proteins are shown in the form of a histogram (Fig. 1) and call for no special comment except to stress that of 74 analyses 26 fell within or below the normal limits.

The histogram (Fig. 2) for the urea levels indicates that 12% of patients had blood urea levels

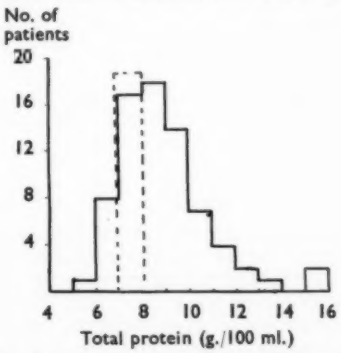

FIG. 1.—Values for total protein (frame=97% normal levels).

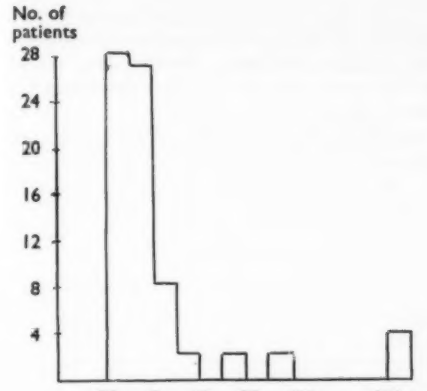

FIG. 2.—Plasma urea concentrations.

above the normally accepted upper limits of 47 mg. for 100 ml. Of the patients with a blood urea over 100, two were admitted with advanced renal failure, the diagnosis of myelomatosis being unsuspected at the time of admission.

Calcium Levels.—Twenty-two per cent. were above the limits of normal, and 10% below normal limits (Fig. 3). The latter all had

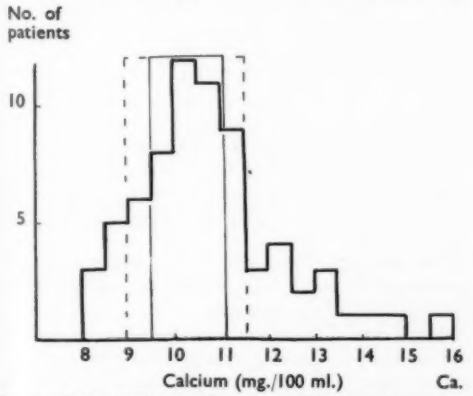

FIG. 3.—Calcium levels (outer frame, – – – 99% normal; inner frame, —— 90% normal).

unequivocal evidence of renal damage. Table I shows the maximum variations in calcium levels observed in five patients on whom serial studies were made with the time interval beween the two readings.

Phosphorus Levels.—Seventeen per cent. fell above upper normal limits, while no levels were

TABLE I

MAXIMAL OBSERVED VARIATIONS IN CALCIUM LEVELS IN FIVE PATIENTS

Code No. in Series	First Observation (mg./100 ml.)	Second Observation (mg./100 ml.)	Time Lapse Between Observations in Months
8	9·0	11·0	6
4	11·8	13·2	4
25	9·6	10·6	8
38	9·4	8·2	3
43	14·0	9·1	10

recorded below 2 mg./100 ml.; 33% had P levels above 4 mg./100 ml. Of these, one, with a level of 12.7 mg./100 ml., was in advanced renal failure. Table II shows renal clearance values on eight patients with values for serum inorganic phosphorus and uric acid obtained at the time the clearance was done.

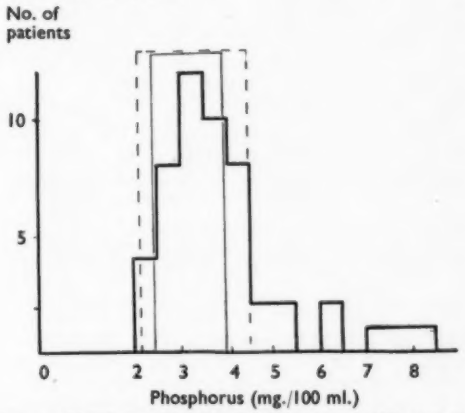

FIG. 4.—Phosphorus levels (outer frame, – – – – 99% normal; inner frame, —— 90% normal).

TABLE II

UREA CLEARANCE VALUES WITH SYNCHRONOUS PHOSPHORUS, URIC ACID, AND TOTAL PROTEIN VALUES IN EIGHT CASES

Case No.	Code No. in Series	P (mg./100 ml.)	Uric Acid (mg./100 ml.)	Total Protein (g./100 ml.)	Urea (mg./100 ml.)	Urea Clearance (% N)
1	38	2·8	6·6	11·3	31	80
2	34	3·5	5·4	11·4	21	80
3	72	3·0	7·0	8·6	50	76
4	1	2·6	5·6	8·4	100	60
5	62	3·2	6·2	8·7	220	45
6	67	6·5	8·4	7·2	305	30
7	44	8·7	5·4	6·7	170	20
8	33	12·7	6·0	9·5	400	8·5

Alkaline Phosphatase Levels.—These were recorded in King–Armstrong units. Of 73 consecutive primary analyses, only one exceeded the absolute upper limits of our normal adult range

and only three fell outside the usually accepted limits (Fig. 5).

Maximal individual fluctuations from follow-up data on five patients are shown in Table III with the time interval between the observations.

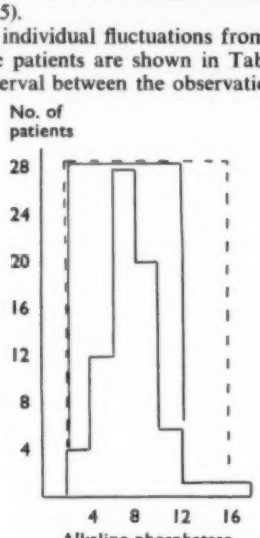

FIG. 5.—Alkaline phosphatase levels (outer frame, - - - 99% normal; inner frame, —— 90% normal).

TABLE III
VARIATIONS IN ALKALINE PHOSPHATASE

Case No.	Code No. in Series	First Observation	Second Observation	Time Lapse Between Observations in Months
1	4	8·3	15·7	6
2	7	6·0	8·5	12
3	38	8·0	12·0	15
4	39	13·0	8·0	6
5	34	6·0	3·0	36

There was no precise relation between the extent of bone involvement as judged radiologically and the phosphatase levels.

Fig. 6 shows initial plasma uric acid levels of 18 patients; eight were above the upper normal limit, and, though the highest levels observed were in a patient with a considerable degree of associated renal damage (see Table II), uric acid levels were above normal in four patients in which there is no clear evidence of renal damage, and of the 18 analyses only two were below the mean of the normal span.

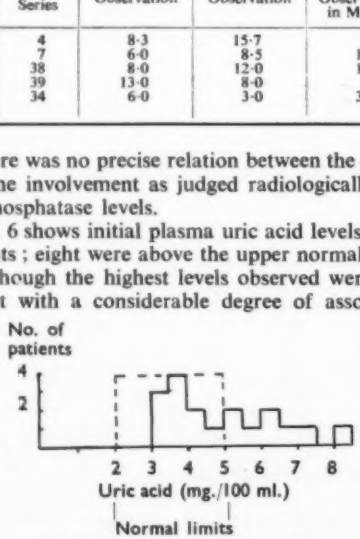

FIG. 6.—Plasma uric acid concentrations.

Cholesterol Levels.—These were analysed in 20 patients. Two were above the upper normal limit of 250 mg./100 ml. One of these was a diabetic of six years' standing.

Discussion

It might be expected that the widespread use of electrophoretic technique, and the bizarre protein pattern it discloses in more than 80% of patients with myelomatosis, would help to establish the diagnosis with greater confidence at an earlier stage in the course of disease. We are not convinced, from a plot of the age incidence of the present series (Fig. 7), that it has materially altered the age at which the diagnosis is established.

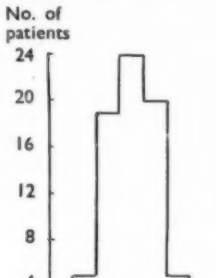

FIG. 7.—Age distribution.

The majority of comprehensive reviews give the incidence of renal impairment as ranging from 50% to 86% of all cases, but do not say at what stage of the disease the assessments were made. However, if a blood urea level above 47 mg./100 ml. be taken as suggestive of renal impairment, only 10 out of 70 patients appeared to have renal impairment when first seen. Two of the patients in the present series were admitted with advanced renal failure, the diagnosis of myelomatosis only being made after admission. There is no doubt that at necropsy a high percentage of patients suffering from myelomatosis would show some renal lesions.

The reported incidence of a significantly raised serum calcium level varies from 28 out of 41

patients examined and reported on by Bayrd and Heck (1947), to 18 out of 35 reported by Lichtenstein and Jaffe (1947). Adams, Alling, and Lawrence (1949) found 24 out of 45 cases had calcium levels above 12 mg./100 ml., while Snapper, Turner, and Moscovitz (1953) report 30 out of 80 cases studied with a serum calcium level above 12 mg./100 ml. In the present series, 13 out of 70 patients had blood calcium levels above 12 mg./100 ml. when first examined. The highest calcium level we have found was 17.0 mg./100 ml., though much higher levels are recorded in the literature, up to 18.8 mg./100 ml. by Robbins and Kydd (1935) and 20 mg./100 ml. by Schittenhelm (1929). Cantarow, in 1935, described a patient whose blood calcium fluctuated between 12.9 mg. and 9.2 mg./100 ml. We have only seen one instance of a fluctuation as great as this in a patient suffering from progressive renal failure, though lesser fluctuations are common.

Unlike patients with active parathyroid adenoma, the serum inorganic phosphorus levels were never below 2 mg./100 ml. Indeed, only four out of 51 initial analyses were below 2.5 mg./100 ml. and at no stage in the condition have we recorded a value below 2.2 mg./100 ml.

At initial examination when less than 15% of the present series had blood urea levels suggestive of some renal damage, 33% had levels of serum inorganic phosphorus above 4 mg./100 ml., suggesting that the raised phosphorus levels often seen in myelomatosis are not the result of renal damage alone.

It is tempting to imagine that they are related to the increased turnover of nucleoprotein and to couple the observation with the relatively high plasma uric acids commonly recorded. Table II shows that three patients of the present series had uric acid levels of 5.4, 6.6, and 7 mg./100 ml. respectively at a time when urea clearance studies suggested an adequate renal function. The levels of uric acid do not show any relation to levels of circulating proteins.

There is little agreement in the literature on the levels of alkaline phosphatase. Bayrd and Heck (1947), in a study of 24 cases, record levels of between 0.8 and 12 Bodansky units, stating that the upper limit of normal would be four Bodansky units. Adams *et al.* (1949), recording the results from 22 patients, found 10 with values above 4 Bodansky units.

In contrast Snapper *et al.* (1953) found only 15 out of 86 patients with serum alkaline phosphatase levels above 12 King–Armstrong units. In the present series only four out of 73 had alkaline phosphatase levels above 12 King–Armstrong units, and only one above 16 King–Armstrong units. We have never examined a patient who at any stage in the progress of the disease had an alkaline phosphatase level above 30 King–Armstrong units, and we consider that a raised alkaline phosphatase is strong presumptive evidence, whatever the radiological appearances, against uncomplicated myelomatosis.

Summary

The initial biochemical findings in 74 consecutive patients, subsequently proved to have myelomatosis, are presented.

Thirteen of these patients had calcium levels above 12 mg. per 100 ml. serum, but in none of these was the serum inorganic phosphorus level below 2.5 mg. per 100 ml. serum.

In 73 out of 74 patients the serum alkaline phosphatase level was within normal limits. It is suggested that to find a markedly raised alkaline phosphatase level is strong presumptive evidence that the patient is not suffering from uncomplicated myelomatosis.

The use of modern methods of protein analysis, while they have helped to establish the diagnosis, have not materially altered the age incidence at which the diagnosis is made.

Our thanks are due to the numerous physicians and pathologists, especially the staff of St. George's Hospital, for the opportunity to examine the patients and make the chemical analysis.

We wish to acknowledge the support of the British Empire Cancer Campaign for defraying part of the expense of this investigation.

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FRACTIONATION OF BENCE-JONES PROTEIN BY STARCH GEL ELECTROPHORESIS

BY

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The development by Smithies (1955a) of starch gel as a medium for the electrophoresis of protein material marked an important advance, as it combines separation by electrical mobility with a form of pore filtration. Smithies (1955b) has been able to obtain 12 or more protein fractions from human serum with his one-dimensional technique and equally remarkable separation has been obtained with animal sera (Ashton, 1957 ; Latner and Zaki, 1957).

Applying this technique to the study of the abnormal proteins of multiple myeloma, Bence-Jones protein, which behaves as a single entity on paper electrophoresis, can often be resolved into two or more components in starch gel. There are a few reports in the literature of the occurrence of more than one abnormal electrophoretic fraction in the urine (Putnam and Stelos, 1953 ; Soulier, 1953 ; Wunderley and Caspani, 1954 ; ten Thije, 1956 ; Vis and Crokaert, 1956 ; Cummings, 1957 ; Gobert-Jones, 1957 ; Owen, 1957 ; Woodliff, Ainsworth, and Flemans, 1958). However, most electrophoretic studies have shown only a single abnormal protein, with or without albumin and normal serum globulins (Gutman, Moore, Gutman, McClellan, and Kabat, 1941 ; Moore, Kabat, and Gutman, 1943 ; Rundles, Cooper, and Willett, 1951 ; Slater and Kunkel, 1953 ; Osserman and Lawlor, 1955 ; Owen and Rider, 1957).

The findings on 13 urines from cases of multiple myeloma, eight of which showed the classical heat reactions of Bence-Jones protein, are reported here.

Material and Methods

Fresh urine specimens and samples of clotted blood were obtained from successive cases of multiple myeloma which satisfied all the accepted criteria for the diagnosis, including typical bone marrow biopsy findings. The urine was examined for the presence of Bence-Jones protein by the classical heat test at a controlled *p*H (Harrison, 1947), the urine being filtered at its boiling point and the filtrate examined carefully for the presence of any precipitate which appeared on cooling and dissolved on reheating. The total protein was estimated by a modified turbidometric method (Kingsbury, Clark, Williams, and Post, 1926), and all specimens, irrespective of the result of the heat test, were subjected to electrophoresis. Before electrophoretic analysis the protein was concentrated where necessary by dialysis of the urine at 4° C. in " visking " tubing against a saturated solution of gum acacia ; the extent of the concentration depended on the initial protein content. Three batches, each of 200 ml. pooled normal urine, which contained no detectable protein by the usual qualitative tests and which were normal on microscopy, were treated similarly for control purposes.

Paper electrophoresis was carried out according to the qualitative technique of Flynn and de Mayo (1951) and starch gel electrophoresis as described by Smithies (1955b). The nomenclature used in conjunction with the latter method is that of Smithies (1955b), extended where necessary to describe bands with differing mobilities. For the purpose of assessing the mobilities of the fractions obtained in starch gels, further analyses were carried out in which the urines and their corresponding sera were run alongside one another in the same gel. In some cases this was done on paper also. To prove that Bence-Jones protein was in fact being fractionated in starch gel, the abnormal band obtained by paper electrophoresis was isolated and run in a gel.

Results

The results are summarized in the Table, and in Figs. 1 and 2 photographs of typical electrophoretic patterns are shown. With normal urines ill-defined patterns were obtained on paper electrophoresis, only albumin and γ globulin being clearly discernible, but in starch gel prealbumins, albumin, post-albumins, and traces of β and γ globulins were demonstrated.

Interpretation of the electrophoretic analyses of the urines from the patients was complicated by the presence of some normal serum proteins.

TABLE

DATA ON URINE AND SERUM PROTEINS FROM 13 CASES OF MULTIPLE MYELOMA

Case No.	Urine					Serum	
	Total Protein (mg./100 ml.)	Result of Heat Test for Bence-Jones Protein	Concentration for Electrophoresis	No. and Position of Myeloma Protein(s) *		No. and Position of Myeloma Protein(s) *	
				Paper Electrophoresis	Starch Gel Electrophoresis	Paper Electrophoresis	Starch Gel Electrophoresis
1	0	—	×550	1–γ	1, slow γ	1, M	2, both slow α_2
2	< 5	—	×250	1–γ	1, slow γ	1, γ	1, fast γ
3	100	Positive	×30	1–γ	1, slow γ	1, γ	1, fast γ
4	385	,,	×10	1–γ	2 {mid γ, fast γ}	0	1, mid γ
5	300	Negative	×17	1–M	4, all $\alpha\beta$	1, M	5, all $\alpha\beta$
6	2,000	Positive	×1	1–M	3 {pre slow α_2, slow α_2, post slow α_2}	1, γ	1, fast γ
7	500	Negative	×6	1–M	3 {β, post β, slow α_2}	1, β	1, $\alpha\beta$
8	150	—	×20	1–M	3 {slow α_2, post slow α_2, post β}	1, γ	1, fast γ
9	120	Positive	×30	1–β	2 {pre fast α_2, fast α_2}	1, γ	1, fast γ
10	500	,,	×6	1–β	2 {fast α_2, β}	1, M	3 {2 $\alpha\beta$, slow α_2}
11	350	,,	×10	1–β	5 {pre fast α_2, fast α_2, 2β, post β}	0	0
12	50	,,	×100	1–β	2 {fast α_2, β}	1, γ	1, fast γ
13	220	,,	×15	1–β	2 {fast α_2, β}	0	0

* Abnormal proteins designated M occupy a position between β and γ bands (Gutman, Moore, Gutman, McClellan and Kabat, 1941).

These were often present in increased quantity and range as a result of the renal damage of the disease. In all the urines, except that of Case 5, these were present at much lower concentrations than the abnormal protein or proteins, and in four patients, Cases 6, 10, 11, and 13, they were not detectable on paper at the concentration of urine examined. In the starch gels, where more material was analysed, traces of albumin and pre- and post-albumins were found in every case and, in some, traces of other fractions also. Recognition of the abnormal protein in the paper electrophoretic pattern presented no difficulty. In the starch gels the decision as to which bands represented Bence-Jones protein was made after considering their relative concentrations, whether they had any counterpart in the serum, and whether they corresponded to fractions found in normal urine. Thus, some Bence-Jones components had the same mobility as a fraction in the serum but could be recognized as abnormal by their density, and others migrated to positions where there was no serum counterpart. That this method of interpretation was reliable was confirmed by the experiments in which the Bence-Jones protein obtained on paper was isolated and run in a starch gel; the bands obtained corresponded exactly with those attributed to Bence-Jones protein on analysis of the whole urine.

Comparison of the electrophoretic behaviour of the abnormal urine proteins on paper and starch gel shows that γ proteins on paper usually correspond to slow γ bands on starch, that β Bence-Jones proteins on paper usually yield a β and a fast α_2 fraction, and M bands (between β and γ) usually yield several fractions with mobilities anywhere between the point of insertion and the β position. The results of the corresponding serum analyses show that γ myeloma globulins on paper invariably correspond to fast γ bands on starch and that M globulins usually correspond to slow α_2 bands; the one β myeloma globulin yielded a band in the $\alpha\beta$ position.

The results of the urine and serum analyses in a given patient show no correlation. In three patients, Cases 2, 3, and 5, the abnormal protein in the urine had the same mobility on paper as the abnormal serum globulin. In starch gel only one of these patients, Case 5, showed abnormal proteins of identical mobility in both, and in that case there was a general proteinuria with a urine pattern virtually identical with the serum. Case 4,

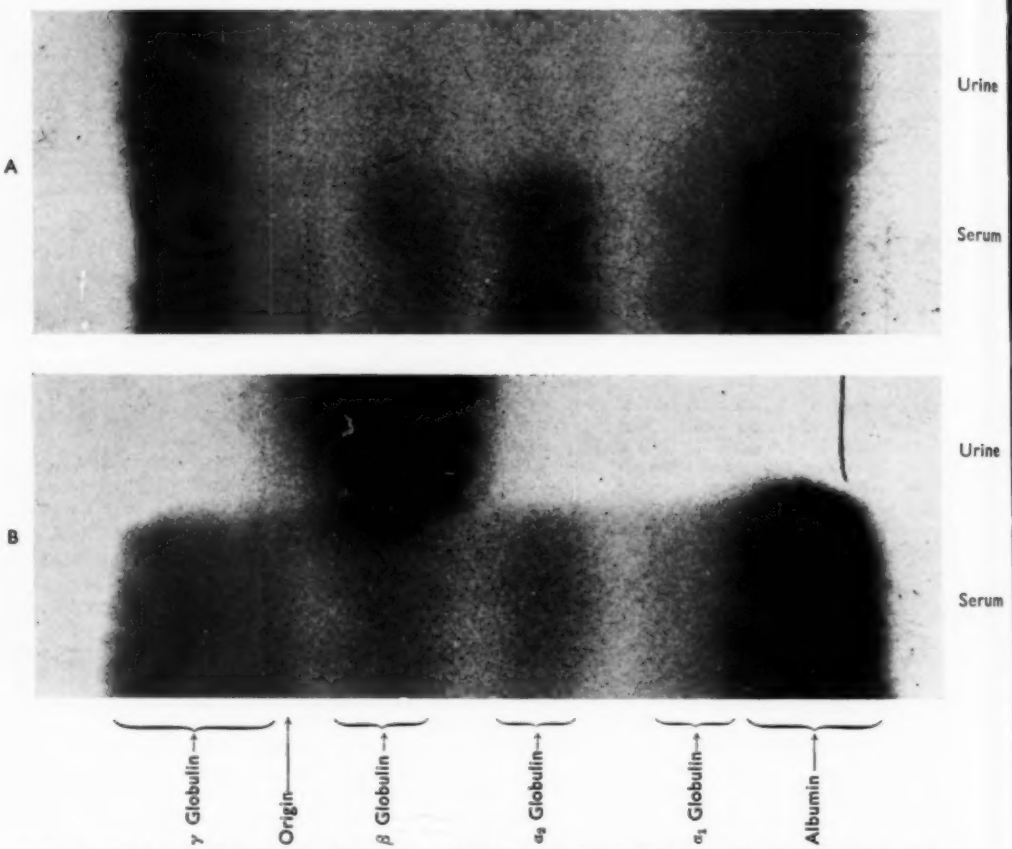

FIG. 1.—Electrophoresis of serum and urine proteins run alongside one another on paper. (A) Case 3 showing single Bence-Jones protein in urine, of same mobility as abnormal serum band, with some normal serum components. (B) Case 13 showing single Bence-Jones protein in urine.

however, had an abnormal band common to both serum and urine when run in starch, without evidence of a gross glomerular leak of protein. This case is also of interest because an abnormal serum band was detectable on the starch when not visible on paper.

Discussion

The above findings concerning the abnormal proteins found in both urine and serum provide further evidence of the complexity of the protein derangement in multiple myeloma.

The demonstration by the starch technique of normal serum components in the urine in all cases is of interest. It may partly explain why complete solution of the urine protein on boiling is so rarely observed, although it is established that on occasion there may be little or no solubility on boiling when virtually pure myeloma protein is in the urine (Osserman and Lawlor, 1955; Vis and Crokaert, 1956; Owen and Rider, 1957; Flynn, unpublished observations).

The results with starch gel electrophoresis provide abundant evidence of the heterogenicity of the abnormal urine protein excreted in myeloma, regardless of its behaviour in the heat test. Thus, 10 out of 13 urine proteins have been shown to consist of two or more components.

The improved resolution in starch gel as compared with paper is due to several factors. First and foremost there is the inherent property of the method itself, combining electrophoresis with a

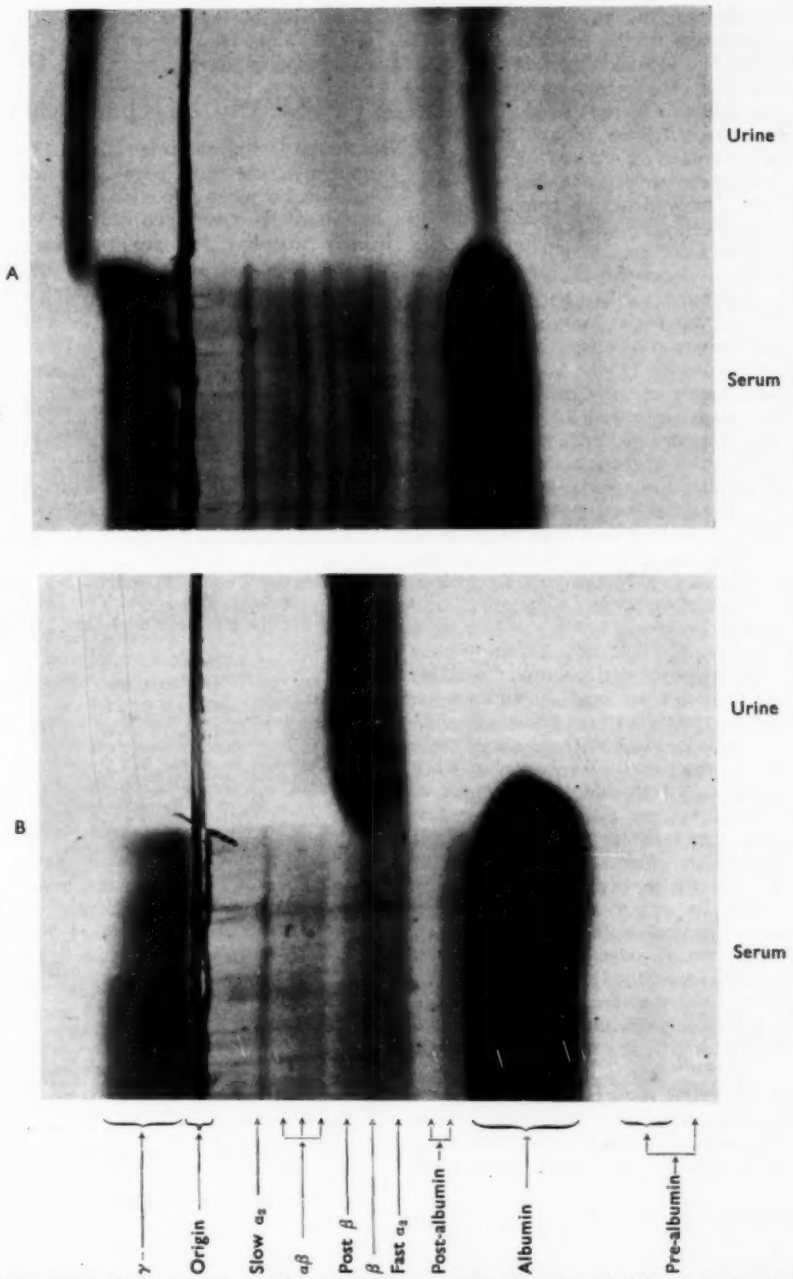

FIG. 2.—Electrophoresis of serum and urine proteins run alongside one another in starch ge (A) Case 3 showing single Bence-Jones protein in urine, of different mobility from abnormal serum band, with some normal serum components. (B) Case 13 showing two fractions of Bence-Jones protein in urine with traces of normal serum components.

form of ultrafiltration, but also the larger sample and the compactness of the bands must play a part. The first of these factors seems to be the most important in the resolution of myeloma urine protein into several fractions. Thus the experiments showing fractionation of the Bence-Jones protein contained on a narrow segment of a paper electrophoretic strip prove that the starch method is not merely detecting components too small to show up distinctly on paper.

Other workers have occasionally found more than one peak on electrophoresis of Bence-Jones protein, as mentioned earlier, but usually only when the *p*H of the buffer was below the isoelectric point (Putnam and Stelos, 1953 ; ten Thije, 1956 ; Vis and Crokaert, 1956). Such a *p*H effect is not the explanation of these results, as the *p*H was 8.6 in our paper runs and 8.2 for the starch analyses. Confirmatory evidence of the heterogenicity of individual Bence-Jones proteins exists in many related studies using salting out, the ultracentrifuge, or immunological methods (Putnam and Stelos, 1953 ; Awapara and Jirgensons, 1954 ; Wunderly and Caspani, 1954 ; Steyn-Parvé and ten Thije, 1955 ; Deutsch, 1955 ; Deutsch, Kratochvil, and Reif, 1955 ; ten Thije, 1956 ; Vis and Crokaert, 1956).

Characterization of myeloma urine protein according to its relative electrophoretic mobility in a starch gel is much more precise than on paper because of the compactness of the bands. Mobilities as such are influenced by the presence of other proteins, which may cause displacement when their concentration is high, but the technique of running serum and urine side by side enables correct identification by noting if a given band is continuous across the junction. An example of this method is shown in Fig. 1 (A) and Fig. 2 (A), where it can be seen that a γ urine protein which appears identical with the abnormal γ globulin in the serum, as judged by its mobility on paper, is distinct on starch. The results on starch gels support the view that the protein appearing in the urine is distinct from the myeloma protein in the serum, at least in the absence of a gross renal leak.

Starch gel has given results which show it to have considerable advantages over other media used for electrophoresis. The demonstration of an abnormal serum globulin in Case 4, when none was detectable by the paper method, shows that this technique is of potential value in the diagnosis of multiple myeloma.

Summary

Serum and urine proteins from 13 cases of multiple myeloma have been examined by electrophoresis on paper and starch gel.

Starch gel electrophoresis has shown that in many patients the abnormal proteins are multiple. Three sera and 10 urines had more than one abnormal component, and apparently homogeneous Bence-Jones protein isolated by paper electrophoresis was shown to consist of several fractions when run in starch gel.

In starch gel abnormal proteins in urine rarely correspond with those in the serum, even when the two have the same mobility on paper.

Starch gel electrophoresis gives better resolution than paper electrophoresis and has potential value in the diagnosis of myeloma.

Work included here was done during the tenure by one of us (E. A. S.) of a grant from the research fund of University College Hospital. We wish to thank also Mr. A. Bligh and Mr. A. C. Lees for the photographs and the clinicians who have provided us with specimens.

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THE EFFECT OF SUNLIGHT AND TEMPERATURE ON THE REDOX POTENTIAL OF LIQUID MEDIA

BY

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It is often found that the characteristic patterns obtained from redox potential measurements in metabolic studies are invalidated by possible variations in the media as well. Consequently the patterns so obtained do not give a true picture of the metabolism, and therefore do not reflect properly the manifestations of life.

Experiments were carried out to show the extent of these variations, and the methods and results of this work are detailed below.

Methods

Measurements of redox potentials were taken with the aid of a Jones *p*H meter. The cells used were *Bact. coli*. The medium was McConkey's single broth. The organisms were incubated at 37° C. for 36 to 48 hours. Two experiments were run simultaneously, one with organisms, referred to as "experimental," the other without them, referred to as "control."

For each potential reading the *p*H was also determined by a rough method, using indicator paper of the appropriate range. The *p*H, as is known, influences the redox potentials. However, according to Hewitt (1950), the introduction of a buffer solution would considerably complicate the situation by bringing a number of unknown factors into the picture.

Results

The readings were plotted in a co-ordinate system, millivolt readings on the ordinate and time on the abscissa. Seven graphs are presented.

Fig. 1 shows the variations in two sterile liquid media (McConkey's single broth), one exposed to daylight and temperature changes (Curve No. 1), the other (Curve No. 2) kept in the dark. One can see the variations in the first part of the redox curve as compared with the slight variations in the one kept in darkness. The temperature was on average 10° C. higher in the exposed one, which also indicates that a considerable amount of heat reached the media (the test-tube was kept at the window). In the exposed media the decrease is more accentuated. This decrease is perhaps due to the beginning of decomposition of the sugar in the media at a considerably higher temperature than the one kept in the dark; the oxidation process may also be due to this higher temperature. The oxidation of the sugar is affected and possibly initiated by the daylight, and the temperature would only have a secondary effect within the range of these temperature variations.

In Fig. 2 the curve marked as "light int." shows the effect of sunshine through the glass in the window, beaker, and test-tube. In this filter system some ultra-violet rays were absorbed, and the heat waves penetrated. The temperature of the media was higher than in the one marked "light external." In this latter one the test-tube was placed outside the laboratory.

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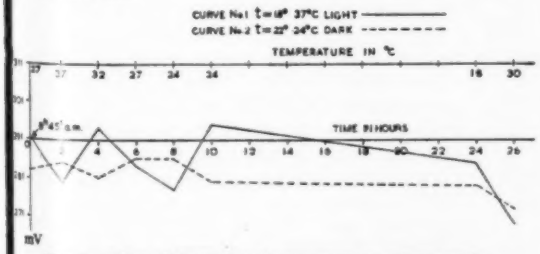

FIG. 1.—Effect of daylight and temperature on culture media.

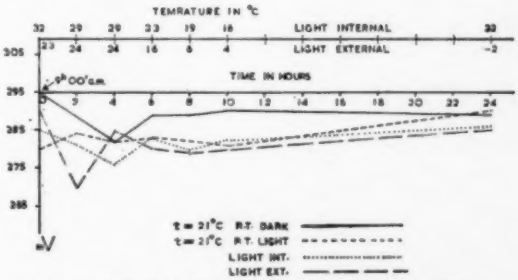

FIG. 2.—Effect of daylight and temperature on culture media.

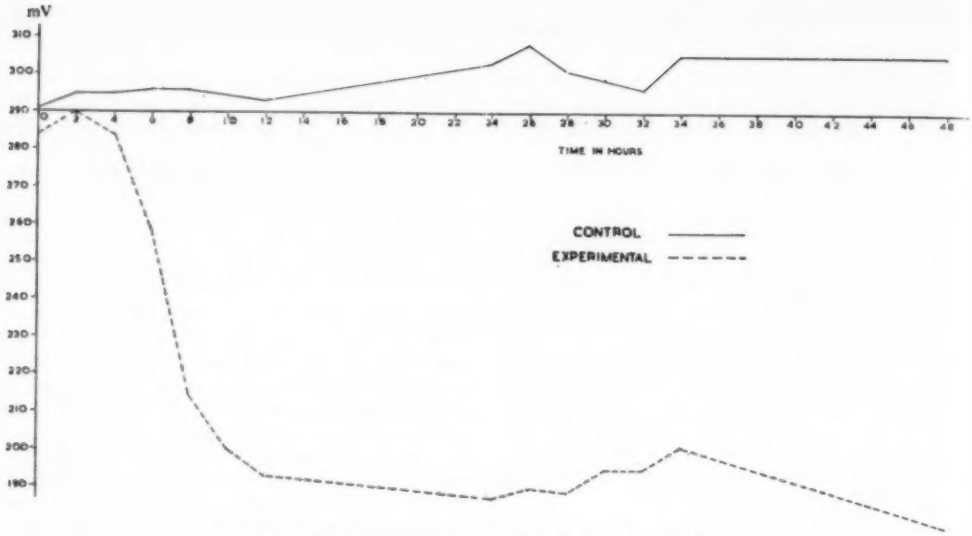

FIG. 3.—Redox potentials in lactose broth in the dark at 37° C.

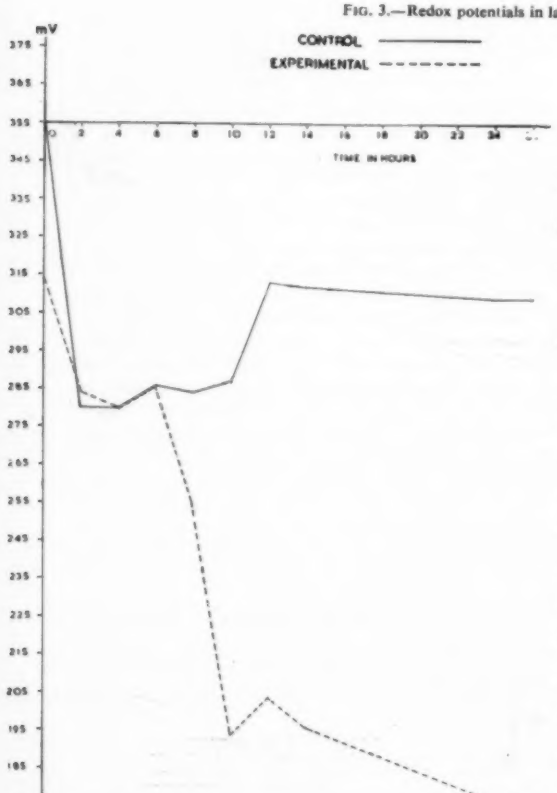

FIG. 4.—Redox potentials in glucose broth in the dark at 37° C.

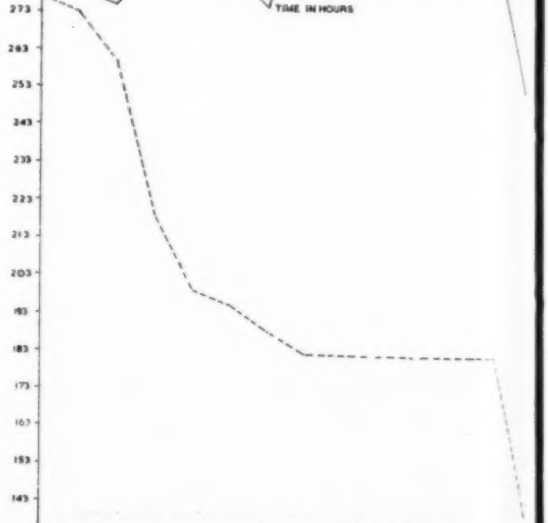

FIG. 5.—Redox potentials in mannitol broth in the dark at 37° C.

The least variation is detected in the medium kept in the dark at a relatively low temperature compared with the curves "light internal" and "light external." Also, when the curves are inspected after a night (14 hours) the tubes exposed to light present variations while the ones kept in the dark are practically unaltered. Variations in all experimental curves in the first two hours are probably due to the temperature differences to which the media are subjected when taken out of the refrigerator.

Figs. 3–5 represent experiments in which the inoculated experimental tubes and the sterile controls were kept in the dark at 37° C. In Fig. 3 the medium was lactose broth and it will be seen that the experimental curve falls steeply in 12 hours, while the control remains more or less steady, hence this is a genuine fall. After that, until 26 hours, the "experimental" hardly changes, while the "control" shows a steady increase. Had the control stood steady, the experimental curve would probably have decreased even more. On the other hand, the increase of the experimental curve between 26 and 34 hours would have been further increased had it not been counteracted by the fair decline of the control curve. The declining slope of the experimental curve between 34 and 48 hours can be considered as a true decline, i.e., the corresponding section of the control curve does not show any variation. However, the decreasing slope of "experimental" in this section does not signify growth since the low *p*H attained in the phase between 12 and 26 hours would not provide the optimum milieu for further growth activities. Also the redox potential of this phase is already fairly low for growing aerobic organisms.

Figs. 4 and 5 show the changes obtained with glucose and mannitol broth. In both cases the changes in the control tube are so great that they interfere significantly with the interpretation of the experimental curves.

Figs. 6 and 7 give the corrected experimental curves of Figs. 4 and 5 respectively, demonstrating rather convincingly how much interference can be attributed to the redox variations in the sterile media and how misleading the interpretation of

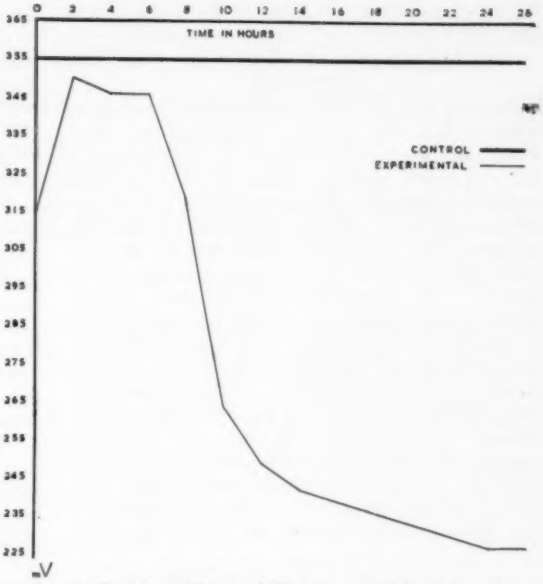

FIG. 6.—Experimental curve of Fig. 4 corrected for changes in medium.

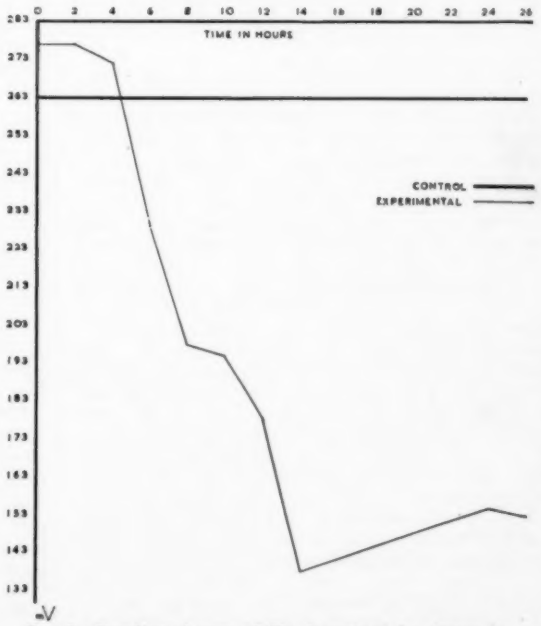

FIG. 7.—Experimental curve of Fig. 5 corrected for changes in medium.

the experimental curves could be if the changes in the media proper were not borne in mind.

Discussion

Proteins are able to carry oxygen by the presence of their SH_2 groups. Sugars in the somewhat increased temperature of the incubator (37° C.) slowly start to oxidize, give off hydrogen, and, by combining with the oxygen, form H_2O_2. When sunshine is present, the formation of H_2O_2 is speeded up. The ultra-violet rays of the sunshine help to form H_2O_2; its concentration depends on the wavelengths and the period of exposure. While the sugar is oxidized, the oxygen, by taking up the liberated hydrogen, is reduced. The presence of this free reduced substance increases the redox potential. This is marked by a steep increase in the positive direction on the graphs. When these substances are protected against sunshine (kept in the dark) the variation in redox potential becomes practically negligible. The substances mentioned above are all present in the common culture media, hence they are likely to produce these reactions and, in so doing, a fairly irregular curve on the redox graphs and considerably interfere with the shape of the experimental curves. In other words, when redox curves are read, the changes produced by the media themselves have to be interpolated in order to be able to reconstruct and obtain the "real" curve and draw the proper conclusions.

Summary

It is found that sterile culture media undergo changes when exposed to alterations in light and temperature. These changes cause considerable variations in the redox curves of the sterile media used as controls. The possible cause of these changes is discussed. Their importance in avoiding misinterpretation of experimental results and drawing false conclusions is pointed out.

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THE FATE OF GLUCOSAMINE HYDROCHLORIDE INJECTED INTRAVENOUSLY IN MAN*

BY

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Glucosamine is known to be a constituent of the numerous mucocomplexes in man. These serve many important functions in the form of intercellular components, cementing substances, lubricants in joint and ocular fluids, and as aids to intestinal motility in the form of mucins of the alimentary tract.

However, apart from a few very incomplete early reports on the fate of glucosamine in biological systems, there is little information available on the metabolism of glucosamine in man. This relative lack of knowledge has inspired the present investigation, namely, the development of an intravenous glucosamine tolerance test. The intravenous rather than the oral route was chosen to eliminate any error due to any breakdown or malabsorption of the compound in the alimentary canal, and because of early reports that oral glucosamine caused diarrhoea.

Most of the information on the metabolism of glucosamine dates back to the earlier part of this century (Offer and Frankel, 1899; Fabian, 1899; Stolte, 1908). Experiments on human subjects carried out at about that time showed that the excretion of glucosamine into the urine began in the second hour after its ingestion and continued for a long period thereafter. Some conflicting results were obtained at the time.

It was not until 1934 that Kawabe, using organic assay, traced the fate of orally and subcutaneously administered glucosamine hydrochloride in normal adult rabbits and found it to be distributed in the liver, kidney, and stomach mucosa as well as in the small intestine and the pancreas. Recently Boas and Foley (1955) injected glucosamine hydrochloride into rats, and found that 80% to 90% disappeared from the blood in 10 minutes. This occurred in the intact animal as well as in one in which the liver or kidney had been excluded from the general circulation, suggesting that the liver did not play a major role in the metabolism of glucosamine. The glucosamine was distributed mostly in those tissues which are rich in extracellular fluid, but the highest concentration was in the liver. Estimation by Boas and Foley (1955) of the total amount of glucosamine left in the animal and excreta at the end of an hour after an intravenous dose of glucosamine hydrochloride showed that approximately 50% of the dose had been metabolized. Their claim that only a small fraction of the intravenously administered dose is excreted in the urine does not agree either with earlier findings in animals or with the present investigation in man.

Methods

A 20% glucosamine hydrochloride solution was prepared for intravenous injection. The 20% concentration was the highest possible for a satisfactory solution. The *p*H of the solution was 2.2, and no attempt was made to buffer it. It was stored at −4° C. At this acid *p*H and low temperature the glucosamine was not broken down. As no information was available on the toxicity of the compound when injected intravenously, preliminary experiments were carried out using rabbits, and the final technique was based on these findings.

The patient fasted from 10 p.m. the previous evening. A fasting sample of blood was taken and divided into two portions, one added to a sodium fluoride-thymol powder, and the other allowed to clot. Then a dose of ½ g./kg. body weight of glucosamine hydrochloride was injected intravenously, taking an average time of 10 minutes.

Clinically the reaction during injection was minimal, the main symptoms being a temporary and slight blurring of vision, a general warmth of the body, and in some instances a "tingling" feeling in the limbs. The pulse and respiration rates showed little or no change.

Blood samples were obtained at 15, 30, 60, 120, and 180 minutes after the injection. Urine was collected four-hourly for 24 hours. Also, in the first

*Submitted in part by S. Weiden for the degree of Ph.D., University of Melbourne.

†Working with the aid of a grant from the National Health and Medical Research Council of Australia.

intravenous tests a 24-hour sample of urine was collected on the day preceding the test as well as on the day of the test and the following one. A few drops of glacial acetic acid were added to the specimens during collection and the bottles were kept in the refrigerator.

Using the sodium fluoride-thymol specimens, protein-free filtrates were obtained and free hexosamine levels determined. The clotted specimens were centrifuged, the serum taken off hydrolysed, and the total hexosamine present determined (Weiden, 1958). The hexosamine content of the urine was determined before and after hydrolysis.

Determination of Free Hexosamine in Blood.—It is necessary to determine the amount of free hexosamine remaining in the blood during the intravenous glucosamine test. A protein-free filtrate of the blood was prepared according to the method described by Somogyi (1945). The hexosamine content of the filtrate was determined by the method previously described (Weiden, 1958).

Estimation of Hexosamine in Urine.—To determine the hexosamine content of urine, 1 ml. of urine was hydrolysed with 1 ml. of 4N-HCl for six hours in a boiling water-bath. After cooling, the sample was neutralized with 4N-NaOH to *p*H 6 using "universal" indicator paper, then diluted with distilled H_2O to a final volume which depended on the concentration of hexosamine present. In order to determine the final volume, in many instances a trial run had to be carried out. The final volumes varied from 10 to 500 ml.

Reports in the literature that it is impossible to determine urinary hexosamine because of interfering chromagens were not substantiated. Interference due to urea, as reported by Lutwak-Mann (1941), was not encountered as the final dilution of the hydrolysate was considerable; this was especially so in the samples obtained immediately following the injection, when, as will be shown later, the greater part of the glucosamine is excreted. In the few instances where an interfering chromagen was obtained, it was due to some medication received by the patient, such as sulphonamides; where possible this treatment was stopped on the day before the test.

Results

Altogether 32 intravenous glucosamine tolerance tests were carried out on 31 patients, including six with uncomplicated diabetes mellitus, three with liver disease, six suffering from carcinoma (one stomach, two lung, one hepatic ducts, one bronchus with secondaries in the liver, and one colon with secondaries in the liver), four with renal disease, and one each with coronary sclerosis, multiple myeloma, lupus erythematosus, Hodgkin's disease, and Sjögren's disease.

Seven patients with quiescent peptic ulcer were included as controls.

Free Blood Hexosamine Levels after Intravenous Injection of Glucosamine Hydrochloride.—Free hexosamine could not be detected in the blood of any of the patients before injection of glucosamine hydrochloride.

After injection there was a rise in the free blood hexosamine; the highest value was obtained at 15 minutes and this was followed by a return towards the normal level in the following specimens. The results are given in Table I.

The seven patients suffering from quiescent peptic ulcer were considered to be normal controls. The three patients with liver disease were suffering from acute infectious hepatitis, chronic infectious hepatitis, and nutritional hepatitis respectively. All three showed poor liver function tests, a raised serum bilirubin level, positive cephalin flocculation, increased γ-globulin level, and, in the latter two, a lowered serum albumin and raised alkaline phosphatase level (Wood, Garlick, Motteram, Weiden, Moore, Mackay, and Turner, 1949). In spite of the poor liver function in these patients, the blood hexosamine levels after the intravenous injection of glucosamine hydrochloride showed the same rise and fall encountered in the control patients.

TABLE I

FREE BLOOD HEXOSAMINE LEVELS AFTER THE INTRAVENOUS GLUCOSAMINE TEST

Diagnosis	No. of Cases	Average Excretion and Range of Free Hexosamine (mg.%)					
		F	Time in Minutes after Injection of Glucosamine Hydrochloride				
			15	30	60	120	180
Normal	7	Nil	63 (38–82)	48 (30–70)	40 (17–54)	24 (9–36)	14 (Nil–21)
Liver disease	3	,,	71 (64–81)	57 (43–71)	41 (32–50)	26 (14–34)	16 (9–21)
Diabetes mellitus with normal renal function	6	,,	64 (15–81)	56 (30–64)	38 (21–60)	27 (13–38)	18 (Nil–29)
Carcinoma	6	,,	67 (23–84)	40 (13–50)	36 (30–41)	26 (18–37)	16 (Nil–33)
Renal disease	4	,,	66 (43–79)	61 (42–73)	56 (40–63)	44 (36–53)	36 (23–46)
Coronary sclerosis	1	,,	28	25	23	14	9
Multiple myeloma	1	,,	60	45	43	23	14
Hodgkin's disease	1	,,	66	56	37	25	20
Lupus erythematosus	1	,,	65	56	46	22	10
Sjögren's disease	1	,,	51	49	37	35	30

The group of six patients with diabetes mellitus were all patients who as yet had received no treatment for their diabetic condition, or in whom the control with insulin and diet had been inadequate in recent months. The renal function in five of these patients was normal as assessed by renal function tests and aspiration renal biopsy (Taft, Finckh, and Joske, 1954). In the remaining patient the renal function was normal and the renal biopsy showed only minimal changes, a minor degree of diffuse glomerulosclerosis and a patchy fibrous and tubular dilatation. Oral glucose tolerance tests carried out on these patients (Fig. 1) showed them to be suffering from well-defined diabetes mellitus. The glucosamine tolerance tests obtained in the same patients are given in Fig. 2 and are compared with the mean normal curve. The free blood hexosamine levels in these patients did not differ from the control group in spite of the inability of these patients to deal with a similar dose of glucose.

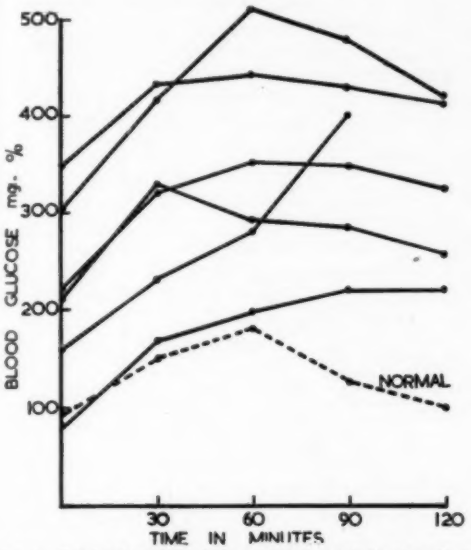

FIG. 1.—Results of the oral glucose tolerance test in the six patients with diabetes mellitus who were also subjected to an intravenous glucosamine tolerance test.

Seven intravenous glucosamine tolerance tests were carried out on six patients suffering from carcinoma. Results obtained were similar to those of the control group.

The group of four patients with renal disease included one with mild chronic nephritis, one with pronounced chronic nephritis, and two with a proved Kimmelstiel-Wilson syndrome consisting of diabetes mellitus, nephrotic oedema, albuminuria, and hypertension. After the injection of glucosamine hydrochloride in these patients there was a high blood hexosamine level over the three hours and a slow return to normal.

Normal levels were obtained after intravenous glucosamine hydrochloride in the patients with coronary sclerosis, multiple myeloma, Hodgkin's disease, lupus erythematosus, and Sjögren's disease.

Effect of Intravenous Glucosamine Hydrochloride on Total Serum Hexosamine.—The total serum hexosamine of all the specimens was

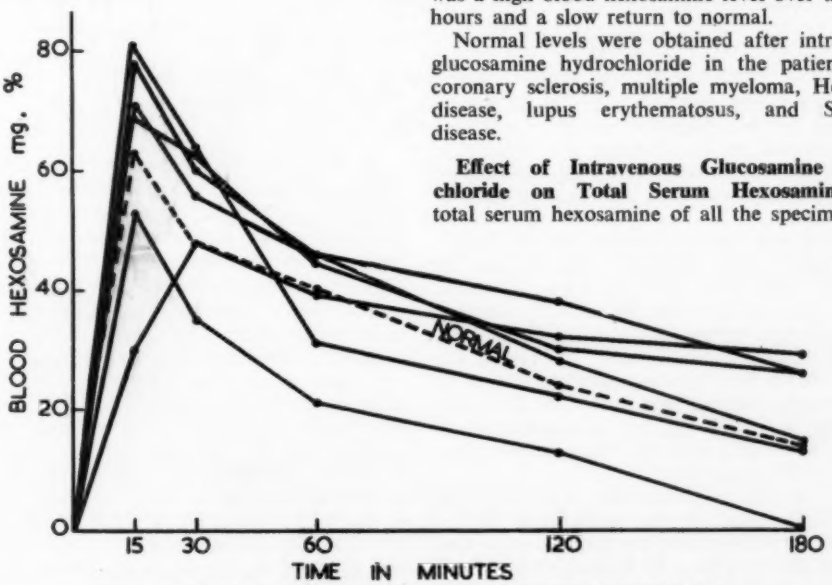

FIG. 2.—Results of the intravenous glucosamine test in the same six patients as in Fig. 1.

determined after acid hydrolysis of the serum (Weiden, 1958). All the patients investigated showed the same rise and fall as for the free hexosamine, and the rise could be accounted for by the amount of glucosamine still circulating in the blood. From this it could be concluded that during the period of the test none of the glucosamine injected had been incorporated into the serum proteins.

Excretion of Glucosamine after Intravenous Glucosamine Hydrochloride.—Nine patients were studied to determine whether all the glucosamine hydrochloride injected intravenously was excreted in the urine on the day of the test. Twenty-four-hour samples of urine were obtained where possible on the day preceding the test, on the day of the test, and on the day following. The results are given in Table II.

TABLE II

HEXOSAMINE CONTENT OF URINE OF PATIENTS IN 24 HOURS PRECEDING, DURING, AND AFTER INJECTION OF GLUCOSAMINE HYDROCHLORIDE

Patient	Intravenous Dose Glucosamine (g.)	Urinary Excretion (g.)		
		Before 24-hour Period	During 24-hour Test	After 24-hour Period
S.C. ..	14	—	3·11	0·21
G.F. ..	12·4	—	5·87	0·61
H.J. ..	16·9	0·14	8·95	—
G.F. ..	20·7	0·42	12·4	0·25
W.J. ..	18·2	0·10	8·87	0·26
B.N. ..	16·6	0·25	8·72	0·26
F.J. ..	14·7	0·25	7·60	0·21
N.J. ..	18·7	—	11·0	0·38
H.J. ..	19·7	0·35	9·04	0·30

Using the values obtained in the 24 hours preceding the test as a base-line, it will be seen that only a portion of the injected glucosamine is excreted and this takes place during the first 24 hours. The renal excretion then falls to the pre-injection level.

After the intravenous injection of the glucosamine hydrochloride, the urine was collected as four-hourly specimens for a period of 24 hours. In most cases the free and the total glucosamine content of each sample was determined and the percentage excreted calculated. The results are given in Table III. It was found that most of the hexosamine excreted was in the free form and not combined. This suggests almost immediate excretion of the glucosamine. The excretion pattern will be discussed later.

The percentage of glucosamine excreted after an intravenous injection of glucosamine hydrochloride is fairly constant; the mean average was 51.3 in the normal group, 58 in patients with liver disease, and 55 in patients with diabetes mellitus

TABLE III

EXCRETION OF GLUCOSAMINE AFTER INTRAVENOUS INJECTION OF GLUCOSAMINE HYDROCHLORIDE

Case No.	Diagnosis	Glucosamine Dose (g.)	Glucosamine Excreted (g.)		
			Free	Total	% (of Total)
1	Duodenal ulcer ..	14·2	—	8·87	48
2	,, ,, ..	15·7	—	7·6	48
3	Gastric ,, ..	16·9	—	8·95	53
4	Duodenal ,, ..	17·4	10·12	9·94	57
5	,, ,, ..	17·9	8·83	8·89	49·7
6	,, ,, ..	18·9	10·86	10·40	55
7	Gastric ,, ..	15·4	6·82	7·37	48
8	Nutritional alcoholic cirrhosis of the liver	20·7	—	12·4	60
9	Chronic infectious hepatitis	19·0	—	11·3	60
10	Acute infectious hepatitis	17·7	—	11·8	66
11	Fatty liver subnutrition	19·7	7·66	9·02	46
12	Long-standing case of diabetes mellitus ..	16·6	—	8·37	50·4
13	Diabetes mellitus duration one year ..	11·6	5·89	6·96	60
14	Moderate severe diabetes mellitus ..	20·6	10·24	10·91	53
15	Short history of diabetes mellitus ..	19·0	10·33	10·1	53
16	Short history of diabetes mellitus ..	17·4	8·78	10·72	61
17	Carcinoma of pancreas with diabetic G.T.C.	18·6	9·94	9·92	53
18	Carcinoma of the lung	12·4	—	5·87	47
19	Second carcinoma of the liver	10·9 16·6	4·36 —	4·25 2·12	39 12·7
20	Carcinoma of main hepatic ducts ..	19·2	—	1·26	6
21	Carcinoma of the stomach	15·8	6·27	6·58	42
22	Terminal neoplasm of the lung	14·2	2·1	2·2	16
23	Duodenal ulcer with slight renal inefficiency	16·2	5·25	5·87	36
24	Chronic nephritis ..	16·4	0·98	1·55	10
25	Diabetic with Kimmelstiel-Wilson syndrome	21·5	6·03	6·64	31
26	Diabetic with Kimmelstiel-Wilson syndrome	18·2	6·97	7·22	39
27	Multiple myeloma ..	16·6	—	8·72	52
28	Hodgkin's disease ..	18·7	—	11·0	59
29	Lupus erythematosus	16·9	—	11·7	69
30	Sjögren's disease ..	15·4	—	5·5	36

without renal disease. It was normal in the patients with multiple myeloma and Hodgkin's disease, raised in the patients with lupus erythematosus, and reduced in those with Sjögren's disease.

The presence of renal insufficiency was the main cause of reduction in the excretion of glucosamine. The patient suffering from chronic nephritis excreted only 10% and the excretion was reduced to a lesser extent in the other patients suffering from renal dysfunction. A pronounced reduction in excretion was noted in some of the patients suffering from carcinoma, and the degree of reduction appeared to depend on the degree of secondary dissemination of the carcinoma.

The excretion pattern over the 24 hours after the injection of the glucosamine hydrochloride was determined for each patient. The greater part of the glucosamine excreted was in the first eight hours after the injection with a return to normal hexosamine level in 12 hours. A moderate reduction in excretion was seen in the patients with carcinoma and a more pronounced reduction in the patients with renal disease (Fig. 3).

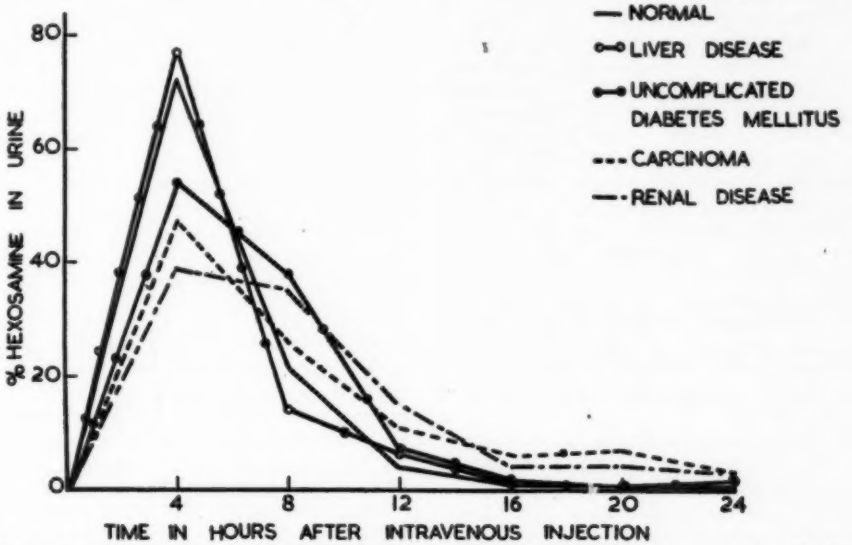

FIG. 3.—Excretion pattern of hexosamine in urine after an intravenous injection of glucosamine hydrochloride. The % hexosamine in urine denotes the percentage of the total amount of hexosamine excreted in 24 hours.

Excretion of Glucosamine in Faeces after Intravenous Glucosamine Hydrochloride. — In one of the control patients (with quiescent duodenal ulcer), the faeces excreted during two 24-hour periods after the intravenous injection of glucosamine hydrochloride were examined for hexosamine content. The entire 24-hour specimens were homogenized in a known amount of water and a sample removed for hydrolysis and hexosamine determination.

The hexosamine content of the first 24 hours was only 56.4 mg. and on the following day 47.6 mg., indicating that, in spite of the large dose (18.2 g.) of glucosamine hydrochloride injected intravenously, very little is excreted in the faeces. In fact the difference between the values in the two specimens cannot be considered significant.

Hexosamine Content of Gastric Juice after Injection of Glucosamine Hydrochloride.—A fine stomach tube was passed in two patients before an intravenous glucosamine hydrochloride test. After removing the fasting gastric contents, the glucosamine hydrochloride was injected intravenously and the entire gastric contents aspirated 30, 60, 90, 120, 150, and 180 minutes later.

To minimize any errors which might occur due to sampling or homogenization, the entire gastric contents obtained for each time interval were hydrolysed, neutralized, filtered, and the hexosamine content of the filtrate determined. The results are given in Table IV.

In both patients there was no significant increase in the hexosamine content of the gastric

TABLE IV

LEVELS OF HEXOSAMINE IN GASTRIC JUICE BEFORE AND AFTER INJECTION OF GLUCOSAMINE HYDROCHLORIDE

Time of Collection	Patient G.F.		Patient W.J.	
	Glucosamine (mg.%)	Glucosamine Secreted (mg.)	Glucosamine (mg.%)	Glucosamine Secreted (mg.)
Fasting	39	12·6	34	12·9
30 minutes after injection	36	11·4	48	12·5
60 " " "	55	8·8	27	1·3
90 " " "	33	5·6	14	3·7
120 " " "	45	14·3	30	14·9
150 " " "	58	13·3		
180 " " "	41	17·4		

juice after an intravenous injection of ½ g./kg. body weight of glucosamine hydrochloride.

Discussion

Previous biochemical studies in man have mostly recorded the direct determination of the mucoprotein content of the serum, or the hexosamine level in the serum as an index of the mucoprotein content. Hexosamine is also a known constituent of mucopolysaccharides which compose the ground substance of connective tissue. Localization of these mucopolysaccharides has been described by Meyer (1938) and Meyer and Rapport (1951). This knowledge has mainly been acquired by their use as substrates for enzyme activity *in vitro*. The biosynthesis of mucopolysaccharides has been followed by the use of radioactive isotopes in bacteria (Topper and Lipton, 1953; Dorfman, Roseman, Moses, Ludowieg, and Mayeda, 1955) and in animals (Schiller, Mathews, Goldfaber, Ludowieg, and Dorfman, 1955).

The present paper offers a new approach to the study of hexosamine metabolism in man by following the fate of hexosamine (glucosamine hydrochloride) when injected intravenously in human controls and patients suffering from a number of diseases.

Before the intravenous injection no free hexosamine could be detected in the blood. After the injection all the patients showed a rise in the blood level and a subsequent fall within three hours. Approximately 50% of the injected glucosamine was excreted in the urine within 24 hours, most being excreted in the first eight hours.

There was no increase in the hexosamine content of the gastric juice or the faeces after the intravenous injection of glucosamine hydrochloride.

Therefore 50% of the dose was unaccounted for and can be assumed to have been taken up by the tissues. The presence of liver disease, uncomplicated diabetes mellitus, or carcinoma did not alter the findings. Results for the total serum hexosamine during the period of the test show no increase in the amount of bound hexosamine, so that it can be assumed that the injected glucosamine has not been incorporated into serum proteins. It may therefore be reasonably assumed that the remaining glucosamine is taken up by the body tissues where it is metabolized or used in the synthesis of mucosubstances.

Kawabe (1934) and Boas and Foley (1955) have shown that glucosamine injected into animals is distributed among the body tissues. Also the breakdown of glucosamine by animal tissues has been observed by Lutwak-Mann (1941), Kawakami (1934), and Watanabe (1936). Leloir and Cardini (1953) found that degradation of D-glucosamine in the cell involves phosphorylation and subsequent deamination. Bacteria (*Esch. coli*) have been shown to decompose D-glucosamine to form ammonia (Oka and Murachi, 1954; Faulkner and Quastel, 1956).

The present study revealed that in the presence of renal disease there was a delay in the return of the blood levels to normal. Also in renal disease there was a pronounced reduction in the amount of glucosamine excreted in the urine, probably due to an inability of the kidneys to excrete compounds with a free amino group as shown by the urea clearance test.

In patients with diabetes mellitus complicated by degenerative vascular disease with hypertentension, proteinuria, and renal insufficiency, the so-called Kimmelstiel-Wilson syndrome, there was a failure of the blood levels to return to normal after an intravenous injection of glucosamine hydrochloride. This was probably the result of renal insufficiency, as similar results were obtained in nephritis not associated with diabetes.

The fact that untreated patients with diabetes mellitus and no renal abnormalities were able to deal with the injected dose of glucosamine hydrochloride would seem to indicate that insulin is not necessary for its immediate distribution. Further evidence supporting this observation has been obtained and will be published later.

The present study in human subjects during health and disease provides evidence of the fate of glucosamine injected intravenously, particularly the rate of clearing from the blood stream and its excretion in the urine; the fate of the glucosamine metabolized by the tissues now awaits confirmation by isotope studies. In most respects these results obtained in human subjects parallel those previously reported for animals by Kawabe (1934), Lutwak-Mann (1941), and Boas and Foley (1955).

Summary

Glucosamine is an important component of the numerous mucocomplexes found in man, such as intercellular components, cementing substances, lubricants in joint and ocular fluids, and as aids to intestinal motility in the form of mucins of the alimentary tract.

In the present study a technique was developed for an intravenous glucosamine tolerance test, and this was carried out on 31 patients, including seven controls.

No free hexosamine could be detected in the blood of the patients before the injection of the glucosamine hydrochloride.

After the intravenous injection of glucosamine hydrochloride in control cases there was a rise in the free blood hexosamine level followed by a return to normal in approximately three hours.

The rise in total serum hexosamine during the test would be accounted for by the free glucosamine present.

Approximately 50% of the glucosamine was excreted in the urine after an intravenous dose of glucosamine hydrochloride.

There was no increase in the hexosamine content of the gastric juice or the faeces after the intravenous injection.

Compared with the control cases there was no change in cases of liver disease, uncomplicated diabetes mellitus, multiple myeloma, Hodgkin's disease, or systemic lupus erythematosus.

In renal disease, including the Kimmelstiel-Wilson lesion of diabetes mellitus, there was a delay in the return of the blood levels to normal.

Urinary excretion in cases of widespread carcinoma and renal disease was reduced.

The distribution of that portion of the injected glucosamine which is considered to be utilized by the tissue is not known and awaits confirmation, possibly by isotope studies.

Our thanks are due to Mr. W. R. Mackay, of the Pharmacy Department of the Royal Melbourne Hospital, for preparing the intravenous solution of glucosamine hydrochloride.

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GALACTOSE AND GLUCOSE TOLERANCE IN NORMALS, THE OBESE, AND POST-GASTRECTOMY PATIENTS

BY

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The interpretation of oral glucose tolerance tests is complicated by varying individual rates of absorption from the intestinal tract. Intravenous tests eliminate the absorption factor, but there is evidence that they may be less sensitive than oral tests in detecting impaired tolerance (Moyer and Womack, 1950).

It was considered that a galactose tolerance test performed in parallel with a glucose tolerance test would indicate the rate of absorption of glucose, since there is evidence that galactose and glucose are absorbed similarly but differ in the manner of removal from the blood.

The rates of absorption of galactose and glucose are independent of the amount of sugar present once a certain concentration is reached (Cori, 1925 ; Groen, 1937). When a mixture of the two sugars is given, the total maximum rate of absorption of the two together approximates the maximum rate of absorption of either when given alone (Cori, 1926).

The actual rate of absorption of galactose is slightly greater than that of glucose, the ratio 110 : 100 being reported by Cori (1925) in rats and 118 : 100 by Groen (1937) in man. No other sugar shows a rate of absorption so closely resembling that of glucose. Fructose is absorbed at half the rate, and "unphysiological sugars," such as rhamnose and xylose, at one-tenth of the rate of glucose (Cori, 1925 ; Groen, 1937). The energy required for absorption (Darlington and Quastel, 1953) and the degree of depression of intestinal absorption by phloridzin (Öhnell and Höber, 1939) are the same for each sugar.

On a normal diet only minute quantities of galactose can be demonstrated in blood or urine, but when more than 20 g. of galactose is given by mouth the sugar can easily be demonstrated in both the blood and urine of most individuals so tested (Harding and Van Nostrand, 1929).

In thyrotoxicosis oral galactose and glucose tolerance tests show impairment although intravenous tests are normal, findings which are attributed to an increased rate of absorption of all sugars in the small intestine (Althausen, Lockhart, and Soley, 1940 ; Barnes and King, 1943). Thyroxine has been shown to have such an effect in animals (Althausen and Stockholm, 1938).

Hypothyroidism (Stenstam, 1946), pernicious anaemia (Stenstam, 1946), sprue, and vitamin B deficiency (Beams, Free, and Glenn, 1941) are all conditions in which oral galactose tolerance tests show flat curves. The flat curves are attributed to impaired absorption of galactose (Beams *et al.*, 1941 ; Stenstam, 1946) and are paralleled by similar curves in oral glucose tolerance tests performed in the same conditions. Normal or impaired tolerance curves are found when glucose or galactose is administered intravenously.

Removal of galactose from the blood is largely effected by the liver (Bollman, Mann, and Power, 1935), and diseases causing impaired liver function are associated with reduction of galactose tolerance. Bauer (1906) was the first to describe the use of galactose in a test of liver function, and based his index of tolerance on the amount excreted in the urine after oral administration. More recently most workers have preferred to use blood levels (Althausen *et al.*, 1940 ; MacLagan, 1940 ; Stenstam, 1946).

Galactose tolerance has been investigated in diabetes mellitus by Kosterlitz (1933) (seven cases), Althausen *et al.* (1940) (15 cases), and MacLagan (1940) (six cases). In only one case was tolerance found to be impaired. Alsev (1952) estimated lactate and pyruvate levels after galactose administration in 10 normals and 10 diabetics. Levels rose in both groups to the same degree and it was concluded that galactose metabolism proceeded normally in diabetics.

Although the removal of galactose from the blood by the liver seems unaffected by insulin, transport across cell membranes can be increased by it (Levine, Goldstein, Huddlestun, and Klein,

1950; Park and Johnson, 1955). In these experiments a high dosage of insulin has been employed; in those based on a perfusion technique the concentration of insulin used has been as much as 1 unit/ml. When this is compared with the daily requirements of less than 1 unit/kg. in the pancreatectomized man (Goldner and Clark, 1944; Ricketts, Brunschwig, and Knowlton, 1945) and when the results of galactose tolerance tests in diabetics are considered, it seems reasonable to conclude that the effect of insulin has no significance in the clinical assessment of galactose tolerance.

On the evidence available it seemed possible that galactose tolerance curves would provide the necessary evidence on the rate of glucose absorption in patients with normal liver function. In this paper are reported the results of parallel glucose and galactose tolerance tests in normal, obese, and post-gastrectomy subjects, and the significance of the findings is discussed.

The investigation was extended to obese subjects because of the reported high incidence of obesity in newly diagnosed diabetics (Joslin, Root, White, and Marble, 1952) and because of our own finding of a high incidence of obesity in patients with symptomless glycosuria and borderline glucose tolerance curves. Post-gastrectomy patients were investigated because they have been considered to show an early rapid absorption of glucose (Smith, Fraser, Staynes, and Wilcox, 1953) and should therefore provide suitable material for the investigation of the idea that galactose tolerance tests will indicate the rate of glucose absorption.

Material

A series of 37 "normal" subjects (aged 16–45 years) was investigated with both glucose and galactose tolerance tests. All were ambulant, apyrexial, and receiving no medication. Twenty-six were laboratory staff, seven were psychiatric outpatients, and the rest were suffering from minor eye or E.N.T. complaints. Their weights ranged from 112 to 204 lb. (82 to 115% expected weight).

Seven post-gastrectomy patients (aged 30–52 years) were similarly investigated. All except one were fit and were either free from or only suffered to a minimal degree from the symptoms which commonly follow this operation. The exception was under treatment for pulmonary tuberculosis.

Both tests were also performed on 18 normal obese individuals aged 17–47. The criteria of obesity were (1) weight exceeding that expected for height, age, and sex by more than 15%; (2) clinical appearance of obesity. Employing these criteria it was found necessary to create a "borderline obese" group for subjects who either exceeded 115% expected weight but did not appear obese, or, being below this figure, did appear obese. A further six subjects were included in this group, four for the former reason and two for the latter.

The 18 obese subjects varied in weight from 120 to 166% of that expected for height, age, and sex. Only one of these was referred because of obesity; he otherwise presented no abnormal symptoms or physical signs. Four were in-patients being treated for minor localized disorders of skin or eye. One was a short-stay patient in the psychiatric department. The remaining 13 were specially asked to attend, their obesity being noted on examination of medical records at two R.A.F. stations.

All subjects except two in the "borderline-obese" group were male.

Methods

A glucose tolerance test was performed on all cases in the morning after an overnight fast, following three days' preparatory diet containing 300 g. carbohydrate each day with normal fat and protein. The diet on the day of the glucose test was adjusted to conform with the diet of the preceding three days. The galactose test was performed similarly on the day following the glucose test. During the whole period the galactose intake was controlled to 5 to 10 g. daily by restricting milk to ½ to 1 pint daily. Smoking was prohibited on the morning of the tests, and during each test the patients were sitting in a quiet room on their own. None of the patients experienced any difficulty in drinking the sugar solution and none complained of any nausea.

Performance of Glucose Tolerance Test.—The subject emptied his bladder. Duplicate fasting blood specimens were taken. Fifty grams of glucose were administered dissolved in 250 ml. of water flavoured with 1 ml. of tinct. auranti B.P.C. Blood samples were taken for two hours at 15-minute intervals from the normal and obese subjects and at 30-minute intervals from the post-gastrectomy patients. Capillary blood was used and "true" glucose was estimated by a modification of the Folin and Wu method (Dische, 1955). The fasting urine and specimens at one and two hours were tested for glucose with Benedict's reagent and for ketone bodies with Rothera's reagent.

Performance of Galactose Tolerance Test.—A venous sample of blood was taken from the patient and he was given 40 g. of galactose in 250 ml. of water flavoured with orange essence. Warming was required to speed the solution of the galactose, but when administered it was at room temperature. Capillary blood specimens were taken at 15-minute intervals for two hours.

Estimation of Blood Galactose.—Galactose, like glucose, is a reducing agent, and all the methods described for glucose estimation utilizing this quality may be employed for galactose. The problem in esti-

mating blood galactose levels is to distinguish reduction due to galactose from that due to glucose. The most widely used technique has been the destruction of glucose before direct estimation of galactose. Yeasts are employed to destroy the sugars selectively; nearly all varieties of ordinary baker's yeast will readily destroy glucose and leave galactose intact (MacLagan, 1940). Allowance must be made for the non-sugar reducing substances in blood.

A number of difficulties arise in using a fermentation method. Yeasts may degenerate and there may be incomplete destruction of glucose. Occasionally yeasts may be encountered which will destroy galactose; such yeasts may be produced with ease in the laboratory (Harding and Grant, 1933). Yeasts have reducing substances adherent to them, which may be completely removed by three washings in water, but on occasion, in our experience, they persist even in spite of many further washings. There is also the possibility that the yeast will contribute some substance which will affect the reducing power of galactose.

In order to guard against these contingencies a modified technique based on King (1951) was used. A venous sample of blood is obtained as the fasting specimen and three samples of it are diluted in sodium sulphate-sodium tungstate solution. The first acts as a blank. Galactose is incorporated into the diluent of the second and third samples so as to create internal standards of 50 and 100 mg./100 ml. Use of such internal standards guards against and, where changes are small, compensates for all the eventualities discussed above.

By experiment it was found that galactose solutions in water readily deteriorate but those in saturated benzoic acid keep indefinitely. The presence of benzoic acid in no way interferes with the action of yeast. A blood glucose concentration of 3,000 mg./100 ml. was completely destroyed in 10 minutes when benzoic acid was present in saturated solution. In the method employed the concentration of benzoic acid in the standards was 1/30 of this.

Although an arteriovenous difference has been demonstrated by Harding and Grant (1933), capillary and venous samples of blood have been employed in

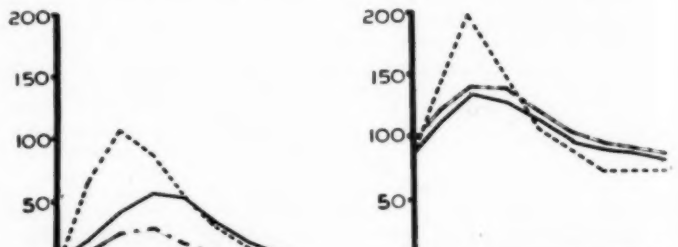

FIG. I.—Composite blood galactose and blood glucose tolerance curves in normal and obese individuals and in post-gastrectomy patients. Ordinates: level of blood sugar in mg./100 ml. Abscissae: time in hours after ingestion of sugar.

TABLE I

PEAK BLOOD GALACTOSE LEVELS IN GALACTOSE TOLERANCE TEST IN NORMAL, OBESE, BORDERLINE OBESE, AND POST-GASTRECTOMY SUBJECTS

Type of Subject	Number of Cases	Mean Age in Years	Mean % Expected Weight	Mean Galactose Peak (mg./100 ml.)	S.D. Galactose Peak (mg./100 ml.)	Range Galactose Peak (mg./100 ml.)	Mean Time of Peak (min.)
Normal:							
Including subject 52	37	23	101	65	29·6	15–152	53
Excluding subject 52	36	23	101	62	24·8	15–110	53
Post-gastrectomy ..	7	41	84	114	12·8	97–130	34
Borderline obese ..	6	28	117	40	26·0	19–90	58
Obese ..	18	28	137	31	22·8	0–76	41

TABLE II

RESULTS OF GLUCOSE TOLERANCE TESTS IN 37 NORMAL CONTROLS

	"True" Capillary Blood Glucose		
	Mean (mg./100 ml.)	Range (mg./100 ml.)	Standard Deviation (mg./100 ml.)
Fasting	87·8	65–107	8·6
Peak	138·9	112–166	14·4
Two-hour	81·1	54–124	12·9
Time of peak ..	35 min.	15–60 min.	11·3 min.

TABLE III

COMPARISON OF NORMAL GALACTOSE RESULTS IN DIFFERENT SERIES

Worker	Year	Number in Series	Interval between Samples (min.)	Highest Peak (mg./100 ml.)	Average Peak (mg./100 ml.)
Kosterlitz	1933	11	30	63	
Harding and Grant	1933	16	15	65	
Nissen	1937	20	15	108	48
Althausen *et al.* ..	1940	10	30	31	19
MacLagan ..	1940	50	30	81	
Stenstam	1946	20	20	108	58
This series ..		37	15	152	65

galactose tolerance tests indiscriminately (MacLagan, 1940). Capillary levels have been used for galactose determinations throughout the present study.

The details of the method are shown in the Appendix.

Results of Galactose Tolerance Tests

Composite curves showing the mean galactose values for the normal controls, post-gastrectomy patients, and the obese are shown in Fig. 1. The important values with their statistical analyses are given in Table I. One subject (No. 52) showed an elevated galactose tolerance curve with a peak value greatly exceeding those obtained in the remainder; it was greater by three standard deviations than the mean of the peak values obtained in the remaining 36 subjects. The glucose tolerance result was within normal limits. A repeat galactose test showed a similarly elevated curve. Physical examination and liver function tests failed to reveal any abnormality. Stenstam (1946) reported similar findings in two fit young women. The results are shown in Table I, including and excluding subject 52. All statistical calculations of significance have been based on the figures including subject 52. Galactose indices (sum of all readings) were calculated but were found to be so closely correlated with peak values that for the sake of simplicity only peak values are reported.

The peak values of the post-gastrectomy series were significantly higher than those of the normals ($P<0.001$).

The peak values of the obese series were significantly lower than those of the normals ($P<0.001$), and the borderline obese group all showed low normal peaks except one.

The data on the normal and obese series have been examined statistically for association with body weight within each group. The height of the peak was found to be independent of body weight, but in the normal series the time of the peak appeared to be earlier, the greater the body weight ($r=-0.42$, $P<0.02$).

Results of Glucose Tolerance Test

Composite glucose tolerance curves in the normals, post-gastrectomy patients, and the obese are shown in Fig. 1.

TABLE IV

RESULTS OF PARALLEL GALACTOSE AND GLUCOSE TOLERANCE TESTS IN 37 NORMAL SUBJECTS

Case No.	Age	Weight	Expected Weight	% Expected Weight	Galactose Tolerance Test (mg./100 ml. Blood at 15-min. Intervals)								Glucose Tolerance Tests (mg./100 ml. Blood at 15-min. Intervals)								
					15	30	45	60	75	90	105	120	F.	15	30	45	60	75	90	105	120
1	34	120	146	82	10	23	41	43	31	15	7	3	90		108	120	156	136	100		80
2	29	156	150	104	21	32	42	54	17	5	0	0	79	118	138	109	97	94	71	71	82
3	19	172	160	107	12	18	13	19	14	2	2	0	94	108	112	106	103	97	94	97	91
4	16	112	114	99	15	23	28	48	64	15	3	0	76	106	120	123	126	123	112	103	97
5	20	145	136	107	2	38	60	67	52	19	7	3	82	133	160	145	124	112	66	60	54
6	19	170	161	105	12	46	67	81	69	49	22	12	92	100	121	155	163	121	92	70	76
7	21	146	144	101	19	43	66	51	17	4	0	0	87	113	135	107	94	91	94	94	86
8	22	171	173	99	9	8	15	13	2	2	0	0	84	102	127	112	94	88	86	78	86
9	21	154	170	91	17	50	69	57	29	13	7	0	86	105	130	133	99	107	89	84	86
10	19	154	146	105	18	48	77	78	54	34	17	8	87	91	145	145	109	88	84	92	89
11	19	140	160	88	4	13	27	31	30	24	7	4	85	112	136	136	112	99	97	97	84
12	26	150	164	92	18	47	88	80	22	6	6	0	84	126	126	120	117	83	80	68	61
13	19	154	154	100	32	77	102	104	66	31	15	15	84	85	125	120	95	83	69	74	82
14	26	128	150	85	42	70	110	89	41	10	0	0	92	115	133	105	98	75	74	69	74
15	19	160	140	114	20	42	67	71	32	10	5	0	93	115	140	125	100	98	90	100	74
16	19	141	138	102	15	32	53	46	24	0	0	0	91	103	133	140	130	120	95	72	57
17	20	129	140	92	0	2	13	2	0	15	2	0	107	110	118	100	83	80	93	95	85
18	35	178	156	114	12	43	77	39	23	12	0	0	96	138	128	117	106	89	76	83	84
19	45	150	158	95	10	68	93	105	79	41	28	15	96	140	166	140	115	89	82	71	71
20	39	188	168	112	9	45	84	91	38	16	9	6	96	137	158	150	121	72	58	67	80
21	16	175	154	113	21	55	45	13	19	12	16	16	103	130	157	141	120	104	91	90	67
22	21	155	153	101	26	44	70	45	20	16	0	0	82	104	123	124	111	84	71	77	77
23	40	141	153	92	7	9	9	15	25	10	0	0	88	91	137	136	136	111	98	105	95
24	22	173	150	115	6	33	21	9	2	0	0	0	77	91	127	115	114	91	93	90	83
25	20	138	138	100	30	48	90	77	42	16	12	0	106	115	124	117	101	86	78	76	74
26	20	138	144	96	8	50	52	49	55	28	17	7	88	114	134	104	97	86	80	83	79
27	22	161	154	104	18	64	72	65	56	22	5	7	88	109	153	152	122	106	106	103	76
28	18	160	144	111	27	61	52	44	32	15	8	5	84	106	131	125	108	112	115	121	124
29	19	132	134	99	48	77	92	87	66	37	14	10	88	109	128	128	119	124	118	111	103
30	23	170	164	104	27	46	61	43	26	4	0	0	82	109	149	128	105	85	82	70	73
31	22	135	139	97	13	41	48	51	34	18	5	0	97	115	139	165	103	103	76	76	73
32	26	177	156	113	3	29	41	41	24	9	12	3	88	94	136	151	112	91	87	82	73
33	18	204	181	113	10	17	9	0	0	0	0	0	83	133	114	105	103	76	76	75	73
34	18	143	144	99	14	38	66	58	27	9	0	0	71	117	123	146	129	117	115	100	96
35	22	135	144	94	11	32	48	71	77	62	42	23	65	77	100	100	120	106	97	83	93
36	23	157	151	104	72	72	85	85	45	16	0	0	86	114	129	122	117	90	88	83	76
37	18	157	158	100	39	122	148	152	134	90	37	22	91	114	157	160	117	86	97	92	86

The results in the normal series are shown in Table II.

In the normal controls but not in the obese a significant inverse relationship ($r = -0.37$, $P < 0.05$) was found between the peak and two-hour value. All features of the tolerance curve, fasting, peak, two-hour values, and time of peak, were examined for a relationship with body weight. No correlation was found between weight and fasting, peak, and two-hour values in either the normal controls or the obese. In the normal controls there was a highly significant correlation between weight and time of the peak, the greater the weight the earlier the peak ($r = -0.48$, $P < 0.01$). This relationship was paralleled by that seen in the galactose tolerance tests. No explanation can be offered for this phenomenon.

The post-gastrectomy subjects all showed peak values over 170 mg./100 ml. Their fasting levels were all normal except one (108 mg./100 ml.), and their mean two-hour value was 73 mg./100 ml., the highest being 83 mg./100 ml.

The borderline obese group all gave results within normal limits.

Seven of the frankly obese subjects showed some abnormal feature in their curves. Six had a fasting level above 107 mg./100 ml. (none in the control series), three had abnormally high peaks, and two showed abnormally high two-hour values. Three of the remaining 11 had peaks higher than 160 mg./100 ml. compared with four of 37 normals.

The full data on all subjects included in this investigation are given in Tables I–VI.

Constancy of the Glucose Tolerance Curve

Gross discrepancies between repeat tolerance curves have been reported by Lennox (1927) and

TABLE V

RESULTS OF PARALLEL GALACTOSE AND GLUCOSE TOLERANCE TESTS IN OBESE AND BORDERLINE SUBJECTS

Case No.	Age	Weight	Expected Weight	% Expected Weight	Galactose Tolerance Tests (mg./100 ml. Blood at 15-min. Intervals)								Glucose Tolerance Tests (mg./100 ml. Blood at 15-min. Intervals)								
					15	30	45	60	75	90	105	120	F.	15	30	45	60	75	90	105	120
Eighteen Obese Subjects																					
38	47	189	156	121	19	36	54	35	10	0	0	0	95	160	170	156	135	91	65	68	82
39	19	189	150	126	0	0	14	14	11	0	0	0	94	92	117	137	150	119	119	77	81
40	21	284	168	169	5	4	8	0	0	0	0	0	93	158	153	121	110	79	81	70	71
41	34	191	142	134	5	26	51	22	4	0	0	0	114	138	162	165	117	94	85	94	88
42	26	209	161	129	25	70	76	20	9	0	0	0	91	107	134	120	86	86	92	75	87
43	41	206	169	122	7	13	26	6	0	0	0	0	97	137	121	109	123	113	70	70	70
44	39	188	149	126	7	21	24	24	10	0	0	0	117	118	137	134	134	123	120	113	96
45	35	228	171	133	22	35	7	0	0	0	0	0	111	119	158	176	155	115	118	100	95
46	19	218	165	132	0	4	0	0	0	0	0	0	84	108	129	97	85	99	90	79	69
47	23	189	136	139	27	47	54	47	14	5	0	0	89	140	164	150	113	91	81	81	72
48	25	225	160	141	0	0	0	0	0	0	0	0	73	82	83	83	83	93	86	93	92
49	42	240	187	128	10	16	24	10	5	0	0	0	86	105	113	167	143	104	77	75	76
50	18	255	158	161	24	40	36	13	8	0	3	0	108	109	116	91	91	88	85	88	85
51	17	194	161	120	0	51	70	50	19	8	3	0	121	121	165	173	145	118	99	87	75
52	19	187	142	131	5	10	12	10	0	0	0	0	96	127	132	145	118	118	122	116	144
53	39	244	163	149	12	17	39	24	11	0	0	0	80	112	123	150	129	117	100	93	79
54	23	202	148	137	0	10	0	0	0	0	0	0	96	144	147	117	105	75	73	64	76
55	19	264	159	166	13	16	19	12	4	0	0	0	112	123	153	171	182	171	171	169	152
Six Borderline Obese Subjects																					
56	27	210	181	116	12	12	17	22	24	10	4	0	91	103	115	109	104	111	98	109	92
57	18	129	112	115	17	29	42	43	28	8	14	4	86	109	127	133	125	120	116	111	84
58	18	194	158	122	4	7	9	27	15	6	0	0	86	90	99	103	96	96	90	69	67
59	19	216	180	120	10	20	36	26	6	3	0	0	84	113	138	145	132	116	112	102	85
60	44	234	206	114	12	13	19	9	5	0	0	0	87	119	132	129	123	109	104	102	85
61	42	152	133	114	12	65	88	90	27	13	5	4	87	121	152	165	121	87	86	87	85

TABLE VI

PARALLEL GALACTOSE AND GLUCOSE TOLERANCE IN POST-GASTRECTOMY PATIENTS

Case No.	Age	Weight	Expected Weight	% Expected Weight	Galactose Tolerance Tests (mg./100 ml. Blood at 15-min. Intervals)								Glucose Tolerance Tests (mg./100 ml. Blood at 15-min. Intervals)								
					15	30	45	60	75	90	105	120	F.	15	30	45	60	75	90	105	120
62						120		51		13		3	76		178		110		69		62
63	35	147	188	78	67	115	95	57	28	7	0	0	73	137	173	132	92	53	53	59	72
64	30	152	156	97		128		41		16		0	78		186		81		77		74
65	46	144	174	83	54	97	87	49	10	0	0	0	108		192		110		82		73
66	42	140	181	76	66	111	88	36	19	9	7	0	94		222		121		69		70
67	41	129	156	86	79	79	81	130	90	46	14	9	101		172		122		86		73
68	52				56	100	84	38	16	8	0	0	99		244		103		66		78

Freeman, Looney, and Hoskins (1942). Himsworth (1935) and Goto (1955) have on the contrary shown identical curves in repeat tests.

Eight subjects have been submitted to repeat tests. One was a "normal obese" individual and showed an impairment of tolerance, and the remaining seven were laboratory staff included in the normal control series. Although some showed close agreement between curves, others showed marked discrepancies, but, apart from one high fasting level and one secondary peak at one and three-quarter hours, all curves in the seven normal controls remained well within normal limits (Fig. 2).

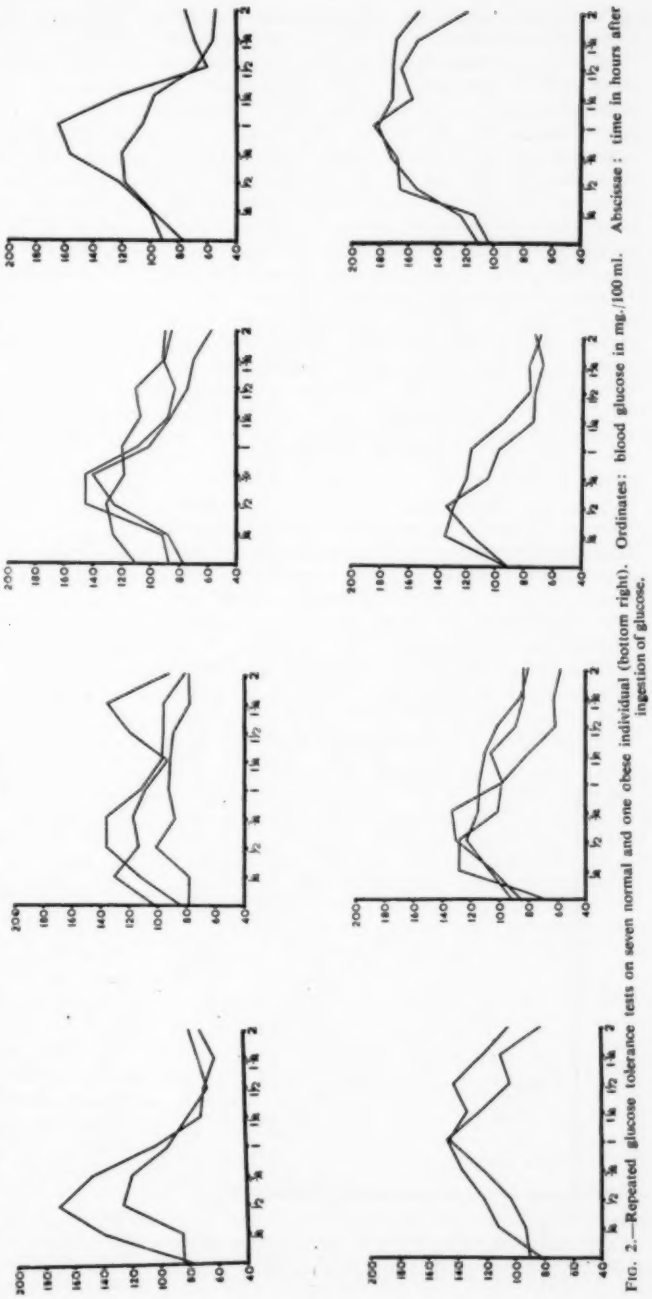

FIG. 2.—Repeated glucose tolerance tests on seven normal and one obese individual (bottom right). Ordinates: blood glucose in mg./100 ml. Abscissae: time in hours after ingestion of glucose.

Discussion

The galactose results in normal subjects are compared in Table III with other normal series where a 40 g. dose of galactose was used. The great variation between workers can probably be accounted for by the techniques of estimation. The results of this series accord closely with those of Stenstam (1946). He found the range of peak values to be 18–108 mg./100 ml. (15–110 mg./100 ml. in this series), the average peak 58 mg./100 ml. (62 mg./100 ml.), and the average time of the peak 50 minutes (53 minutes).

When allowance is made for the differing details of performance and techniques of blood glucose estimation, the glucose tolerance results are found to be in accord with those of other "normal" series (Evensen, 1942; Mosenthal and Barry, 1950; Moyer and Womack, 1950).

The inverse relationship shown between the peak and the two-hour value would appear an important feature of the normal

glucose tolerance curve. In application a peak value slightly exceeding normal limits combined with a low normal two-hour value or a two-hour level just above the accepted upper limit, but following a low peak concentration, may be regarded as normal, whereas a "high normal" peak and a "high normal" two-hour value may indicate impaired tolerance. An examination of the glucose tolerance tests performed in post-gastrectomy patients yields evidence in support of this.

Knowledge of the peak concentration is thus shown to be of value in assessing tolerance contrary to recently expressed opinions (Mosenthal and Barry, 1950; Moyer and Womack, 1950; Friend, 1951; Duncan, 1952). The peak value would seem to be a reasonably constant feature of the tolerance curve, the coefficient of variation (standard deviation expressed as a percentage of the mean being 9.6% as compared with 9.8% for the fasting values and 15.8% for the two-hour values).

Statistical examination shows no correlation between peak values of glucose and galactose in the normal control series. Close correlation, however, could not be expected since even when repeat tolerance tests are performed with the same sugar considerable variation is encountered. Furthermore, each value is the resultant of two main factors, the rate of absorption and the rate of removal from the blood. The rate of absorption of galactose is thought to be similar to that of glucose, but its removal from the blood is not influenced by the same factors as affect the removal of glucose.

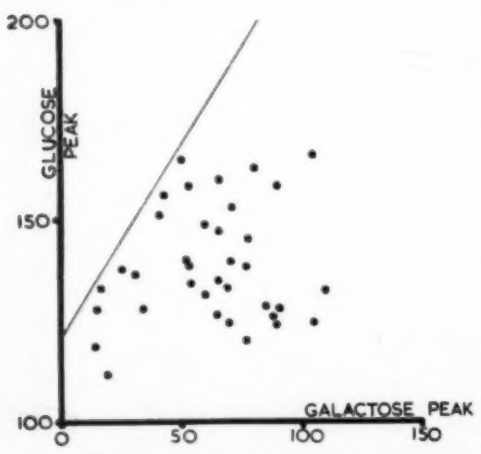

FIG. 3.—Scatter diagram of glucose and galactose peak values in normal individuals. (Blood sugar values in mg./100 ml.) Low galactose peaks were invariably associated with low glucose peaks.

If the normal subjects with low galactose peaks are considered, it is found that all except one had a low glucose peak, although high galactose peaks are not necessarily associated with high glucose peaks (Fig. 3). This finding is in accord with the principle that when hepatic function is normal the galactose tolerance curve gives an indication of the rate of absorption of glucose. The homeostatic mechanism for glucose in the normal is able to compensate to greater or lesser degree for rapid absorption with more rapid removal, and, where absorption is fast, an active mechanism may result in a comparatively low peak. Where absorption is slow, little homeostatic activity is required to achieve a low peak, and hence except in subjects with impaired tolerance low glucose peaks are consistently found.

In patients who have had a partial gastrectomy, the gastro-jejunal anastomosis results in glucose solutions rapidly reaching the small intestine, which is the main site of sugar absorption. This is the explanation given for the consistently high glucose tolerance peak levels seen in most of these patients. Seven cases were examined, and, as expected, very significantly higher galactose tolerance peak values were found than in the normal control series. This suggests that peak galactose levels do mirror the rate of glucose absorption, particularly when the rate of absorption is considerably altered from the average normal. The gastrectomy cases were all under expected weight (mean 84% expected weight, range 76%–97%), and for this reason a dose-body weight relationship could be suggested as partly responsible for the finding of high peak galactose levels. In view of the lack of correlation between galactose peak level and body weight within the series of normal controls and obese subjects, this appears unlikely. An analysis of covariance of galactose peak and body weight over all the groups studied shows that the galactose peaks in the post-gastrectomy patients are in fact higher than would be expected from their body weight.

Compared with the results found in the normals and post-gastrectomy series a marked divergence of glucose and galactose results is seen in the obese. Slow absorption of glucose in the obese would tend to mask impairment of tolerance assessed by the oral test. If the low galactose figures do in fact reflect slow absorption of glucose, then it seems probable that a high proportion of these "normal" obese individuals had impaired glucose tolerance, since they reach the

borderline of abnormality in spite of slow absorption. Here again a possible alternative explanation would be a galactose dose-body weight relationship, but as with the gastrectomy cases this is unlikely and for the same reason.

It seems that obesity (excessive adipose tissue) as opposed to actual body mass is the determining factor in the low galactose peaks observed in the obese. It is possible that adipose tissue can remove galactose from the blood in a manner comparable with the liver. Bansi (1953) has shown in certain obese subjects that glycogen is laid down excessively in fat after the ingestion of glucose. Nevertheless slow absorption seems the more likely cause of the low galactose peaks and this receives support from certain other observations. Strang and McClugage (1931) investigated the specific dynamic action of a protein meal in normal and obese individuals. They showed that the total heat effect was similar but that in the obese the effect was delayed, the peak being lower and persisting over a longer period. Booth and Strang (1936) showed that in normals there was a rise in skin temperature after a protein meal. In the obese this was delayed and diminished. All these findings could be explained by a slow rate of absorption in the obese.

Summary and Conclusions

The assessment of glucose tolerance using the oral test is complicated by the variable rate of absorption which may occur in the intestinal tract. The galactose tolerance test has been proposed as a means of indicating the rate of absorption of glucose.

Glucose and galactose tolerance tests have been performed on 37 normal controls, seven post-gastrectomy patients, 18 obese, and six borderline obese subjects. A modified method of blood galactose estimation has been employed.

Normal values in oral glucose and galactose tolerance have been reported and attention has been drawn to the inverse relationship which normally exists between peak and two-hour blood concentrations in the glucose test. Some relationship was noted between glucose and galactose curves performed in the same individual, but this was not a close one. Repeat glucose tests in the same individual have been shown to reveal marked variations.

Since a fixed dose of sugar was employed in both tolerance tests the tolerance data were examined for variations with body weight, but none was found. A surprising inverse relationship was shown to exist between the time of the peak and body weight in both glucose and galactose tolerance tests. No explanations could be offered.

Tolerance tests in post-gastrectomy patients revealed "lag" glucose and "lag" galactose curves; in each case they closely resembled one another. These findings were considered to support the proposed use of the galactose tolerance test to indicate the rate of absorption of glucose.

Seven of 18 obese subjects showed some abnormality in the glucose tolerance test. There was a very marked trend for galactose tolerance curves to be low. It is suggested that in obesity the rate of absorption is slow in the intestinal tract and that this tends to mask impaired glucose tolerance when it is assessed using the oral test.

APPENDIX

Estimation of Blood Galactose

The method used was a modification of that of King (1951).

Reagents.—The following are required:

(1) *Sodium Sulphate-Sodium Tungstate Solution.*—This is made up of 88 ml. 3% sodium sulphate, and 12 ml. 10% sodium tungstate.

(2) *Yeast Suspension.*—Yeast (less than two weeks old preserved in the refrigerator), 2 g., is washed three times in distilled water and suspended in 10 ml. of 3% sodium sulphate.

(3) 7% *Copper Sulphate Solution.*

(4) *Galactose Standards.*—(*a*) This is a stock solution (prepared every three months) of 1.1429 g. of galactose in 1,000 ml. saturated benzoic acid. (*b*) The "100 mg." working standard is freshly prepared before use by adding 5 ml. of the stock standard to 100 ml. of sodium sulphate-sodium tungstate solution. (*c*) The "50 mg." working standard is a 1:1 dilution of the "100 mg." standard in sodium sulphate-sodium tungstate solution.

(5) *Folin and Wu Copper and Phosphomolybdic Reagents.*—These are prepared as in the blood glucose method of Dische (1955).

Method.—(1) The samples are arranged as follows:

	Test	Blank	Standards	
			"100"	"50"
Sodium sulphate-sodium tungstate solution	3·5 ml.	3·5 ml.		
100 mg. standard solution ..			3·5 ml.	
50 " " " ..				3·5 ml.
Fasting blood		0·2 ml.	0·2 ml.	0·2 ml.
Test specimen	0·2 ml.			

(2) Add 1 ml. of yeast suspension, mix, and incubate in a water-bath at 37° C. for 15 minutes.

(3) Remove, precipitate proteins with 0.3 ml. of 7% copper sulphate solution and centrifuge.

(4) Remove 2 ml. of the filtrate into Folin and Wu tubes and proceed as for blood glucose estimation (Dische, 1955).

It is found that there is a linear relationship between reduction and galactose concentration between 10 and 100 mg. Below 10 mg. the relationship follows a curved line from the blank value to intersect the straight line drawn through 50 and 100 mg. standard readings at 10 mg./100 ml. galactose.

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KLEBSIELLA SEROTYPES IN INFECTIONS OF THE EAR AND UPPER RESPIRATORY TRACT

BY

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The carbohydrate nature of the Klebsiella capsule was first demonstrated by Toenniessen (1912, 1921). However, as recently emphasized by Tomcsik (1956), he was unaware of its antigenic reactivity. The serological specificity of capsular polysaccharide was described by Heidelberger and Avery (1923, 1924). On the basis of this work, Julianelle (1926) demonstrated three capsular types, A, B, and C, and a heterogeneous group X, including several untyped strains. The source of Julianelle's strains was mainly sputum from cases of human pneumonia. Type C has subsequently been associated with rhinoscleroma. Gastings and Snijders (1936) later extended these types to six, by describing three additional types, D, E, and F, isolated from cases of ozaena. In Kauffmann's (1949) classification, these types are designated 1–6 and subsequently discovered types by higher numerals. Including the three types recently described by Ørskov (1955a), the present number of serotypes has reached a total of 72. Although these have been isolated from a wide variety of sources, there is general agreement that the lower types, especially 1–6, are far more frequently recovered from the respiratory tract than elsewhere in the body. Henriksen (1954a), analysing 46 sputum strains, found that 54% were lower types. Similarly Edwards and Fife (1955) showed that 53% of 199 sputum and 90% of 20 nasopharyngeal strains belonged to types 1–6. There are, however, obvious regional differences in the distribution of individual types, as, in Copenhagen, Ørskov (1955b) found that the classical respiratory types 1–6 were only represented by 15.6% of 102 sputum strains.

It is proposed here to present a serological analysis of Klebsiellae isolated from common infections of the ear and upper respiratory tract and also to assess their frequency of incidence.

Material

The 50 strains of Klebsiellae studied in this series were isolated in the course of routine bacteriological examination of the material received in the Pathology Department over a period of 14 months (October, 1955–November, 1956). Strains were selected for study on the basis of their biochemical criteria, irrespective of whether or not they exhibited the mucoid colonial morphology of typical Klebsiellae. These criteria included such recently developed investigations as the cyanide, gluconate, and α amino-acid decarboxylase tests and are discussed elsewhere (Epstein, 1958a).

Serotyping Technique

Before serotyping, all strains were subcultured for 18 hours on lactose agar plates (lactose 0.5%, peptone 0.5%). As emphasized by Duguid (1948) and Edmunds (1954), the high carbohydrate nitrogen ratio of this medium is favourable to capsule formation, yet tends to discourage the production of excessive slime. Wet collargol mounts were prepared from each strain and examined with a 1/6 objective, to determine the presence or absence of capsules. No attempt was made to type

TABLE I

BACTERIOLOGICAL ANALYSIS OF OTITIS AND SINUSITIS

	No. of Cases	Bacteriology (%)								
		Staphylococcus pyogenes	*Streptococcus pyogenes*	*Streptococcus pneumoniae*	*B. proteus*	*Ps. pyocyanea*	*Escherichiae*	*Klebsiellae*	*Haemophilus*	Mixed and Miscellaneous
Acute otitis media ..	534	25·3	20	7·9	6·4	9·9	5·5	1·4	3·5	20·1
Recurrent otitis media	269	32·4	14·5	7·9	7·3	4·6	3·3	1·3	7·9	20·8
Chronic otitis media	562	28·4	6·0	1·8	27·6	11·5	7·6	1·8	1·6	13·7
Otitis externa ..	183	34	5·0	0·9	22·4	11·6	4·5	1·4	—	20·2
Sinusitis	324	19·4	11	16·4	6·5	2·6	7·8	3·1	11	22·2

acapsulate strains. Capsulate strains were typed by capsular swelling tests, using pure capsular sera types 1–10 inclusive. The term "capsular swelling" is used here without prejudice, as Klieneberger-Nobel (1948) and Duguid (1951), among other workers, have shown that there is no true swelling when capsulated organisms are placed in their homologous sera. In fact a precipitin reaction occurs at the surface of the capsule, which then becomes visible due to an increase in its refractility.

Edwards and Fife (1952) have stressed that antibody deviation may occur with high antigen concentrations. For this reason, saline suspensions were adjusted to an approximate density of 10 organisms per 1/6 objective field. Further, as recommended by these authors, only freshly prepared suspensions were used.

Loopfuls of sera and suspension were mixed and mounts examined after five-minute intervals. Capsular titres were not estimated. The serological identity of each type was checked by absorption tests with homologous strains from the National Collection of Type Cultures. Sera were absorbed with strains under test for 30 minutes at 37° C. The subsequent recovery of sera by centrifugation was facilitated by the use of minimal quantities of antigen. Mutual cross-absorption tests, the final proof of identity as emphasized by Edmunds (1957), were not performed.

TABLE II
DESCRIPTION OF KLEBSIELLA STRAINS ANALYSED

Strain	Source	Isolation in Culture	Colonial Morphology	Antecedent Antibiotics	Serotype
	Ear (30)				
3383	Acute otitis media	P	+	Penicillin (1)	1
121	,, ,,	M	+	—	1
124	,, ,,	M	+	—	1
743	,, ,,	M	+	Penicillin (1)	1
2983	,, ,,	M	−	,, (5)	1
1262	,, ,,	M	−	,, (2)	U.T.
1472	,, ,,	P	−	,, (7)	U.T.
378	,, ,,	P	−	Terramycin (7)	U.T.
3469	,, ,,	P	−	Penicillin and aureomycin (1)	U.T.
2147	,, ,,	M	−	—	A.C.
610	,, ,,	P	+	Aureomycin (2)	1
Recurrent					
3486	,, ,,	P	−	—	5
3722	,, ,,	M	−	Penicillin (2)	U.T.
3872	,, ,,	P	−	,, (?)	A.C.
180	,, ,,	M	+	—	1
Chronic					
1608	,, ,,	P	+	—	1
1993	,, ,,	M	+	Penicillin (?)	1
1436	,, ,,	P	−	–	1
1896	,, ,,	M	+	–	1
3373	,, ,,	P	+	Penicillin (1)	1
3599	,, ,,	P	+	—	4
1599	,, ,,	P	−	—	U.T.
3502	,, ,,	M	−	—	U.T.
1100	,, ,,	P	−	—	U.T.
1017	,, ,,	M	−	—	A.C.
1999	,, ,,	M	−	—	A.C.
2066	,, ,,	P	−	Penicillin (4)	A.C.
1144	Otitis externa	P	−	—	1
4019	,, ,,	M	+	Aureomycin (1)	1
3657	,, ,,	M	+	—	U.T.
	Nose and Sinuses (16)				
615	Atrophic rhinitis	M	+	—	3
1770	,, ,,	M	−	—	U.T.
3911	,, ,,	M	−	—	A.C.
1839	Acute sinusitis	P	+	Penicillin (2) and aureomycin (8)	1
87	,, ,,	P	−	Penicillin (7)	U.T.
3518	Chronic sinusitis	P	+	,, (1)	1
178	,, ,,	P	−	—	1
3549	,, ,,	M	+	—	1
3614	,, ,,	M	+	—	2
560	,, ,,	M	−	—	U.T.
1331	,, ,,	M	−	—	U.T.
559	,, ,,	M	−	—	U.T.
2121	,, ,,	P	−	—	U.T.
2770	,, ,,	P	−	—	A.C.
3765	,, ,,	P	−	—	A.C.
2651	Post-nasal space	P	−	Penicillin (?)	2
	Miscellaneous (4)				
3422	Acute tonsillitis	P	+	Penicillin and sulphadiazine (7)	1
2608	Bronchitis	P	+	—	1
1442	,,	M	−	—	1
1445	Acute pharyngitis	M	−	—	U.T.

U.T. = Untypable strains. A.C. = Acapsulate strains. + = Mucoid and tenacious colonies. − = Non-mucoid and non-tenacious colonies. P = Isolated in pure culture. M = Isolated in mixed culture. Figure in brackets denotes duration in days of antibiotic administration.

Results

The majority of strains studied in this series were isolated from cases of otitis or sinusitis, a bacteriological analysis of which is presented in Table I. As can be seen, the approximate incidence of Klebsiellae in this material is 2%. Table II presents a description of these strains with particular reference to their source of origin, antecedent antibiotic history, and serotype. In 48% of cases the strains were isolated in pure culture, in 22% with pathogenic staphylococci, and in the remainder with a variety of other organisms. Table III, based on data derived from Table II, indicates that approximately half of the typed, untypable, and acapsulate strains respectively, were isolated in pure culture. Antibiotics were administered previously in 38% of cases, but in only 12% did the duration of therapy exceed four days. It is of interest to note that 40% of the total number of strains and 73% of the typed strains respectively exhibited a mucoid and tenacious colonial morphology.

TABLE III
DISTRIBUTION OF STRAINS WITH REFERENCE TO STATE OF ISOLATION IN CULTURE

	Typed	Untypable	Acapsulate	Total
Strains isolated in pure culture ..	14	7	3	24
Strains isolated in mixed culture	12	9	5	26
Total ..	26	16	8	50

As shown in Table II, eight strains were acapsulate and 16 untypable with the available sera. Twenty-six strains were typed, of which 21 were found to

belong to type 1. Absorption tests demonstrated the ability of these 26 strains to deplete their type sera of antibody to homologous N.C.T.C. strains.

Discussion

The series presented here is based on an analysis of strains mainly isolated from clinically defined infections of the upper respiratory tract and its extensions. Using sera types 1–10, 62% of the capsulate strains were typable. Henriksen (1954a), on the other hand, using as many as 61 type sera, found that only 54% of 189 capsulate strains could be typed. His series, however, was heterogeneous, and included strains derived from urine, faeces, and water as well as sputum.

The value of Klebsiella typing has recently been challenged by Henriksen (1954b), who stressed that the increase in the number of serotypes has imposed serious practical limitations on this procedure. He also indicated the limited reproducibility of serological results and the increasing number of cross-reactions between closely related types. Edmunds (1957), however, suggested that some of these difficulties may well be due to non-specific reactions. He pointed out that, as Henriksen (1954a) used prolonged immunization courses, there was the possibility of "undue development of antibody to minor antigenic constituents." It is furthermore doubtful whether capsular titrations enhance the specificity of serotyping, quite apart from the additional labour involved. Edwards and Fife (1952) and Ørskov (1955c) are in agreement that absorption tests are of greater value than titrations in the confirmation of serological identity.

It is necessary to effect some compromise between the extreme that dismisses serotyping as impractical and the perfectionist attitude which insists that all 72 capsular sera, albeit in polyvalent pools, be employed and the results qualified by titrations or absorption tests.

Weiss, Eisenberg, Spirack, Nadel, Kayser, Sathavara, and Flippin (1956) recently maintained that types 1, 2, and 4 are rarely found in the respiratory tract, except in association with specific disease processes. They further indicated that the higher Klebsiella types may be found in the throat of healthy persons, and, as will subsequently be pointed out, this may occur especially after narrow-spectrum antibiotic therapy. It would seem reasonable, therefore, to suggest that the routine laboratory restricts typing to a limited number of sera designed to include the common respiratory types. Henriksen (1954b) went so far as to suggest that if a strain isolated from the respiratory tract did not belong to one of the lower types, it was usually safe to assume that it exercised no aetiological role in the disease.

Authors such as Lipman, Coss, and Boots (1948) and Obrinsky, Dormont, Fowler, and Ruhstaller (1950) have minimized the primary role of Klebsiellae as causative factors of disease. They suggest that alleged recent increases in the incidence of Klebsiella infections can be ascribed to antibiotic superinfections. Weiss *et al.* (1956) agreed that antibiotic therapy is often associated with the emergence of Klebsiellae, but pointed out that these often proved to be higher types and of doubtful pathogenicity. Weinstein, Goldfield, and Chang (1954) indicated that superinfection rarely occurs unless antibiotic therapy has been prolonged for more than four days. This would imply that 88% of the strains isolated in this series are unlikely to have been secondary invaders. Furthermore, even in three of the six cases where antibiotics had been exhibited for over four days, the strains isolated proved to belong to lower types.

As indicated in Table III, approximately half of the strains in this series were isolated in pure culture. Even if in some of the remaining cases of bacteriologically mixed infections Klebsiellae did not constitute the predominant pathogens it would be difficult to deny them any aetiological role. It is, moreover, recognized procedure to treat mixed infections with antibiotics to which all isolated pathogens are sensitive, irrespective of the precise quantitative relationships which obtain in primary culture.

The relatively high incidence of acapsulate strains —16% in this material—can be explained by the fact that selection was based on biochemical rather than morphological criteria. In most series the majority of acapsulate strains are automatically excluded from study, as they do not usually produce slimy mucoid colonies. Ørskov (1955c), in a recent investigation of faecal strains, found only two acapsulate out of a total of 76 strains. However, strains producing non-mucoid colonies were excluded from these studies. Bias of this type is equally apparent in Henriksen's (1954a) series. While it is true that such selection is likely to produce an abnormally high incidence of capsulate strains, yet, as emphasized by Edwards and Fife (1952), it must be appreciated that there is no correlation between a mucoid colonial morphology and the presence of capsules. In fact occasional acapsulate strains may be highly mucoid. In Kauffmann's (1949) classification of Klebsiella antigenic patterns, the latter is designated as MO (mucoid, acapsulate with O antigen), in contrast to the classical MKO form (mucoid, capsulate with O

antigen). Kauffmann (1949) also showed that MO, unlike MKO forms, are susceptible to O agglutinins, although, as subsequently indicated by Edwards and Fife (1952), slime (M) and capsular (K) substances are serologically similar, if not identical.

Toenniessen (1915) demonstrated that acapsulate Klebsiellae could be highly virulent and concluded that the association between capsule formation and virulence was fortuitous. Topley and Wilson's *Principles of Bacteriology and Immunity* (Wilson and Miles, 1955) points out that "capsule formation is often associated with, but not essential to, virulence." Furthermore, as subsequently indicated, acapsulate Klebsiellae can act as respiratory tract pathogens in mice. Apart from this, it must be appreciated that three of the eight acapsulate strains in this series were isolated from clinically defined infections in a state of pure culture.

Primary Klebsiella (Friedlander) lung infections are well recognized clinical entities, which have recently been discussed by Erasmus (1956). He suggested that they probably represent a specific form of aspiration pneumonia, related to a precedent pharyngeal or middle ear infection with the same organism. There is also ample experimental evidence proving that Klebsiellae are capable of initiating infections of the respiratory tract and its extensions in animals. Hoyle and Orr (1945) and Sale and Wood (1947) demonstrated the ability of Klebsiellae to produce pneumonia in mice and rats inoculated by intranasal and intrabronchial techniques respectively. Using an intra-oral technique (Epstein, 1958b; Epstein and Stratton, 1958) representative strains of Klebsiellae in the present series and also other acapsulate strains (Epstein and Payne, 1958) have been shown to produce fatal pneumonic infections in anaesthetized mice. Furthermore, Friedmann (1955, 1957), in studies on experimental otitis in guinea-pigs, concluded that Klebsiellae can produce primary infections of the middle ear cleft. Thus both clinical and experimental studies confirm that these organisms may assume the role of respiratory tract pathogens.

Summary

The approximate incidence of Klebsiellae in clinically defined common infections of the upper respiratory tract and its extensions was found to be 2%. Fifty strains of Klebsiellae isolated from these sites were examined. Only 40% of strains exhibited a mucoid and tenacious morphology. Using capsular sera types 1–10, 26 strains could be typed, of which 21 were found to belong to type 1. Of the remainder, 16 strains were untypable and eight acapsulate.

There is suggestive evidence that the majority of these strains represented primary pathogens and not secondary invaders, consequent on antibiotic therapy.

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TECHNICAL METHODS

A Simple Capillary Viscometer

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The plasma proteins are responsible for the viscosity of plasma being greater than that of distilled water. Lawrence (1950) described a method for estimating the total and the differential viscosity of the plasma proteins. Changes in the total plasma viscosity (T.P.V.) have been considered more indicative of organic disease than of elevation of the erythrocyte sedimentation rate (E.S.R.) (Houston, Harkness, and Whittington, 1945; Benson and Goddard, 1954; and others); Lawrence showed that changes in the viscosity of the fibrinogen and gamma globulin fractions were an even more sensitive indicator than changes in the T.P.V. He reviewed the most reliable methods for determining the T.P.V., and gave a description of his modification of the Ostwald viscometer. He stressed the necessity of maintaining a constant temperature (±0.3° C.) for all viscometry. In practice this has been achieved by means of a thermostatically controlled water-bath. The following instrument was designed to dispense with the need for this costly piece of equipment without significant loss of accuracy.

Description of Viscometer

Principle.—A small water-jacket is used to maintain the viscometer at constant temperature. Temperature regulation of the water in the jacket is achieved very simply by the removal of a small volume of water and by the addition of colder or warmer water as necessary to maintain a temperature of 20° C. (±0.3°).

Materials.—The viscometer capillary is of "veridia"* glass 7.5 cm. long, internal diameter 0.5 mm. (±0.01 mm.), external diameter about 4 mm. This is connected by means of rubber pressure tubing to a 1-ml. Ostwald pipette, the end of which is shortened to correspond in external diameter to that of the capillary. The ends are filed smooth and square, and should be touching each other inside the pressure tubing. Marks A and B are etched above and below the bulb (see illustration). A square "perspex" water-jacket (5 × 5 × 16 cm.) is constructed to house the pipette, holes being drilled also for a Van Slyke thermometer reading to 0.2° C., and for

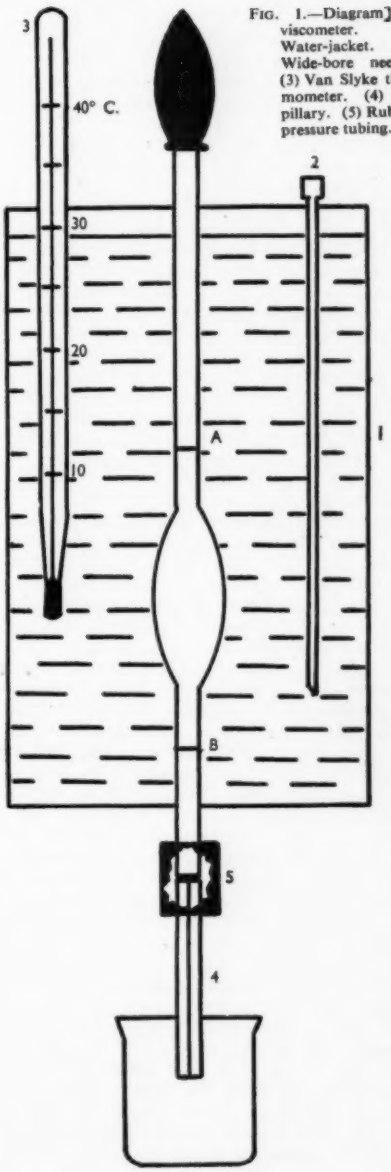

FIG. 1.—Diagram of viscometer. (1) Water-jacket. (2) Wide-bore needle. (3) Van Slyke thermometer. (4) Capillary. (5) Rubber pressure tubing.

* Obtainable from Messrs. Chance Bros., Glassworks, Smethwick, Birmingham, 40.

a long wide-bore serum needle. The whole instrument is clamped to a metal stand. Other equipment necessary is an accurate stop-watch, a 20-ml. syringe, a number of 5-ml. "pyrex" beakers, and some small (1-ml.) rubber teats.

Methods.—Plasma is obtained (as described by Lawrence, 1950) from 5 ml. of venous blood, and pipetted into a "pyrex" beaker. The viscometer pipette is filled by means of a teat through the capillary (which is left *in situ* at all times), to a point well above mark A. If necessary the temperature in the water-jacket is adjusted to 20° C. (±0.3°) using the 20-ml. syringe either with hot tap water or with water that has been refrigerated. Sufficient air is introduced at this operation to ensure thorough mixing of the water in the jacket. As the external temperature is usually close to 20° C., two minutes are sufficient for the temperature of the plasma to stabilize, though in very warm or cold weather three minutes must be allowed for this operation. At the end of this time, the teat is removed and the time taken for the plasma under test to flow between marks A and B is recorded. The mean of two determinations (three if the sample is abnormal) is taken and compared with the (known) time for distilled water (viscosity = 1) under identical conditions. Then, from Poiseuille's law, the relative viscosity of the test solution can be obtained from this comparison:

$$\frac{\text{Time for test solution (in seconds)}}{\text{Time for distilled water (in seconds)}} = \text{relative viscosity of the solution}$$

if the specific gravity of the test solution is ignored (for plasma, this introduces an error of less than 1%).

The sample of plasma is then clotted by the addition of 0.015 ml. (measured in a haemoglobin pipette) of 40% calcium chloride, care being taken to avoid excess, as this inhibits clotting (Lawrence, 1950). After incubation for half an hour at 37° C. serum is expressed from the clot (using a glass rod) and its viscosity is then measured in exactly the same way as that described for plasma. The difference between these two measurements gives the "fibrinogen viscosity." (The other plasma proteins may be precipitated with ammonium sulphate as described by Lawrence, but in this study only fibrinogen viscosities were determined.)

To clean the instrument the pipette is filled and emptied forcibly through the capillary with normal saline solution (by means of a teat). The lower end of the capillary is then attached to a suction pump, and distilled water is poured from a beaker into the top of the pipette. In the same way, the instrument is dried with spirit and acetone. (There is no need to remove the instrument from its stand for these operations.) The instrument should always be cleaned after a sample of high viscosity; also when following the measurement of plasma viscosity by that of serum and *vice versa*. It is not necessary to clean the instrument after the measurement of the viscosity of normal samples of plasma (or serum). The time for distilled water (which should be constant, ±0.1 sec.) should be estimated at the beginning and end of a series of determinations and also if necessary after abnormal samples have been tested.

Results

Blood was obtained from 45 apparently healthy blood donors and medical students. Using Lawrence's method of expressing results, that is, to multiply by 100 and to calculate viscosity differences (i.e., if plasma viscosity is 1.71, then the plasma viscosity difference is 71), the results set out in Table I were obtained for normal total plasma and fibrinogen viscosities.

TABLE I
VALUES FOR PLASMA AND FIBRINOGEN VISCOSITIES FOUND IN 45 NORMAL SUBJECTS

	Plasma	Fibrinogen
Mean	71	14
Maximum	81	20
Minimum	56	5
Standard deviation	7	3·5

Discussion

These results are in agreement with those of other workers determining the plasma viscosity at 20° C. (Miller and Whittington, 1942; Woodmansey and Wilson, 1948). It should be emphasized, however, that such results cannot be directly compared, for each capillary has its own characteristic "shear" for colloidal solutions. Nor is it possible to calibrate this instrument described against Lawrence's viscometer "O," for his measurements are made at 25° C. with correspondingly faster times. If Lawrence's results for plasma are increased by 12.5% (Table II) the factor which he states represents the approximate increase of the values at 20° C. compared with those at 25° C. (the values for fibrinogen are very similar at the two temperatures), it can be seen that they bear a close relationship to the values given in Table I.

TABLE II
THE CORRELATION WITH LAWRENCE'S VALUES

	Lawrence's Values for Plasma	Corrected to 20° C.	Lawrence's Values for Fibrinogen
Mean	58	65	13
Maximum	71	80	19
Minimum	44	50	5
Standard deviation	10		2

The results recorded here apply to this one instrument only, but similar instruments will give similar though not necessarily identical results, the upper limits of normal being obtained from the study of a sufficient number of normal plasmas.

The regulation of the temperature in the water-jacket by hand represents the simplest arrangement that is compatible with accurate work. If many determinations were undertaken, the "perspex" bath could be enlarged and a small thermostat added. The

abolition of the small, though constant, dead space between the end of the Ostwald pipette and the capillary is another possible refinement. However, the experimental error for the instrument as shown by repeat experiments does not exceed ±0.7% (cf. Lawrence's method where the error is ±0.4%). No difficulties were found in maintaining a constant temperature in the water-jacket for the period of the test under conditions of high (or low) external temperature. Owing to the rapid rate of flow of fluid through the capillary, no significant error is introduced because the capillary itself is not surrounded by the water-jacket.

The instrument described has the advantage that, being straight, it is not subject to the filling errors that may occur in U-type viscometers. It is of course liable to such sources of error as air bubbles and particulate matter in the fluid under test, and to timing errors, but these are avoided by good technique as are those caused by haemostasis at the time that the blood is collected. Other types of straight viscometer have been described. Repeat determinations are impossible with the instrument invented by Mann (1948), as the sample is lost in a water-bath. Woodmansey and Wilson (1948) prevented this occurrence by enclosing their viscometer in a test-tube before putting it into a water-bath, but such an arrangement does not dispense with the need for a water-bath. It can be seen that the advantages of employing a water-jacket as opposed to a water-bath are real.

Lawrence (1950) has demonstrated the value of the measurement of the total and differential viscosities of the plasma proteins, and with the capillary viscometer described such observations are possible without requiring elaborate equipment.

Summary

A simple capillary viscometer is described, and the values for total plasma and fibrinogen viscosity found in 45 healthy subjects are recorded.

I should like to thank Dr. J. S. Lawrence of the Empire Rheumatism Council Field Unit, Manchester, for much valuable help and criticism.

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The Determination of Ethylene Diamine Tetra-acetic Acid (E.D.T.A.) in Urine and Serum

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It is now generally recognized that intravenous infusions of ethylene diamine tetra-acetic acid (E.D.T.A.) as the calcium disodium salt readily remove excess lead from the body in plumbism. Up to 4 g. of the salt may be employed at each session. Leckie and Tompsett (1958) have made a study of the use of this substance in plumbism both for therapeutic and diagnostic purposes. It appeared desirable to carry out simultaneous determinations of E.D.T.A. and lead in the urine, since it was found that the excretion of the former may be delayed in the presence of the renal disease which may accompany plumbism. Similar studies would be of value in the assessment of the use of E.D.T.A. orally, concerning which there is little information.

Darbey (1952) has described a method for the determination of E.D.T.A. in urine. Excess of a nickel salt is added, a very stable nickel chelate compound with E.D.T.A. being thereby formed. Excess nickel is removed by precipitation with dimethylglyoxime, the nickel chelate compound remaining in solution. The nickel content of the filtrate, which is a function of the E.D.T.A. content, is then measured. The reagent employed by Darbey for the determination of nickel, which he used directly, is, however, unobtainable. The following is a modification of the method in which the nickel content of the filtrate is determined by the conventional dimethylglyoxime method after destruction of organic matter (cf. Sandell, 1944).

It was considered desirable to measure the E.D.T.A. content of serum. The question of the deproteinization of serum appeared at first to create a problem, but this was overcome by using one of the well-known metal precipitating techniques, nickel being used in place of zinc sulphate. The filtrates usually contain sufficient nickel to combine with the E.D.T.A.

Reagents

(1) 1.33% (w/v) solution of nickel sulphate ($NiSO_4.6H_2O$) in water
(2) Ammonia, sp. gr. 0.88
(3) 1.5% (w/v) dimethylglyoxime in ethanol
(4) Concentrated sulphuric acid, Analar
(5) Perhydrol, Analar
(6) Saturated bromine water

(7) Deproteinizing solutions (serum)

A. 12.5 g. $NiSO_4.6H_2O$, 125 ml. of 0.25 ml. N. sulphuric acid, water to 1,000 ml.

B. 0.75 N. sodium hydroxide

(8) Standard solution of ethylene diamine tetra-acetic acid (1 mg./ml.): 0.1476 g. of $[CH_2.N(CH_2.COOH).CH_2.COONA]_2.H_2O$ is dissolved in water and the volume made up to 100 ml.

Procedure

Urine.—To 10 ml. of urine is added 1.5 ml. of 1.33% nickel sulphate solution and the mixture allowed to stand at room temperature for 10 minutes, then 0.5 ml. of ammonia (sp. gr. 0.88), is added, followed by 1.5 ml. of a 1.5% ethanolic solution of dimethylglyoxime. After standing for 10 minutes at room temperature, the mixture is filtered (Whatman No. 42 filter paper).

One millilitre of the filtrate is measured into a round-bottomed digestion flask followed by 6 drops of concentrated sulphuric acid. Organic matter is destroyed by heating and the addition of perhydrol.

The clear digest is diluted to 10 ml. with water, 5 drops of saturated bromine water added, and the solution well mixed. Four drops of ammonia (sp. gr. 0.88) are added, followed by 0.5 ml. of a 1.5% ethanolic solution of dimethylglyoxime, the solution being shaken after each addition.

A blank (10 ml. water) and a standard (2 mg. E.D.T.A. in 10 ml. water) are prepared at the same time, the complete procedure being carried out. After standing for 15 minutes at room temperature, the colours developed in the standard and unknown are read against the blank at 515 mμ.

Serum.—To 1 vol. serum is added 8 vol. of reagent (7A) followed by 1 volume of 0.75 N. sodium hydroxide. The mixture is allowed to stand at room temperature for 15 minutes and then filtered (Whatman No. 42 filter paper).

Sixteen millilitres of the filtrate are digested with sulphuric acid and perhydrol as described for urine. The nickel content of the digest is similarly determined.

A blank and a standard (200 μg. E.D.T.A.) are prepared at the same time.

Discussion

The results shown in the Table indicate that satisfactory recoveries of added E.D.T.A. could be made from urine and serum.

TABLE

RECOVERY OF E.D.T.A. ADDED TO URINE AND SERUM

	E.D.T.A. Added (mg.)	E.D.T.A. Recovered (mg.)	Recovery (%)
	A. Recovery Added to Urine		
1	0·25	0·22	88
2	0·50	0·47	94
3	0·75	0·69	92
4	1·00	0·95	95
5	1·50	1·52	101
6	2·00	2·08	104

Volume of urine used, 10 ml.
Initial content of E.D.T.A. in urine, nil.

	E.D.T.A. Added (μg.)	E.D.T.A. Recovered (μg.)	Recovery (%)
	B. Recovery Added to Serum		
1	100	92	92
2	200	208	104
3	250	240	96
4	300	320	107
5	400	390	98
6	600	590	98

Volume of serum used, 4 ml.
Initial content of E.D.T.A. in serum, nil.

Substances in urine which may simulate the chelating property of E.D.T.A. were found never to amount to more than 27 mg./litre.

In the examination of the E.D.T.A. content of urine by the above procedure, Beer's law is followed up to concentrations of 0.5 g./litre urine. When concentrations above this figure are encountered, suitable dilutions must, as a preliminary, be made. This presents no difficulty in the examination of 24-hour collections of urine, since such should, under most conditions, contain practically all the administered dose, provided an intravenous infusion has been employed. In other cases some degree of trial and error will be necessary.

This problem will not arise in the case of serum examinations, since concentrations seldom exceed 10 mg./100 ml.

Summary

A method is described for the determination of E.D.T.A. in urine and serum.

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A Note on Urea Estimation by Diacetyl Monoxime

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(RECEIVED FOR PUBLICATION NOVEMBER 19, 1957)

Rosenthal (1955) has described in great detail a method using diacetyl monoxime which is generally satisfactory for the routine estimation of urea in blood and urine except that complete colour development requires prolonged heating in boiling water. This disadvantage can be overcome by the use of a pressure cooker.

The tubes of reaction mixture are immersed in a beaker filled with hot water to a level above that of the liquid in the tubes. The beaker is placed in a domestic pressure cooker which has been warmed during the preparation of the reaction tubes, heated until all air is expelled, and heating continued for 10 minutes at 15 lb. steam pressure. Heating is then stopped, the pressure allowed to fall to zero, and the tubes removed and cooled.

Further heating produced no increase of colour. The colour produced appears more stable. There was approximately 3% fading in one hour under fluorescent light and 12% on standing for two days.

One of the reagents (arsenic acid in concentrated hydrochloric acid) is unpleasant and difficult to dispense accurately and the final reaction mixture has a sickly smell. The range, for 0.1 ml. of blood and 10 ml. final volume, is 0–200 mg. urea/100 ml.

On the credit side, in comparison with the usual urease-nesslerization methods are greater sensitivity and colour stability as well as greater reagent stability and a shorter time for the analysis.

REFERENCE

Rosenthal, H. L. (1955). *Analyt. Chem.*, **27**, 1980.

A Rotary Drier for Use in the Dry Ashing Method for Estimating Plasma Protein-bound Iodine

P. LOCKWOOD

From the Mental Hospitals' Group Laboratory, West Park Hospital, Epsom, Surrey

(RECEIVED FOR PUBLICATION SEPTEMBER 24, 1957)

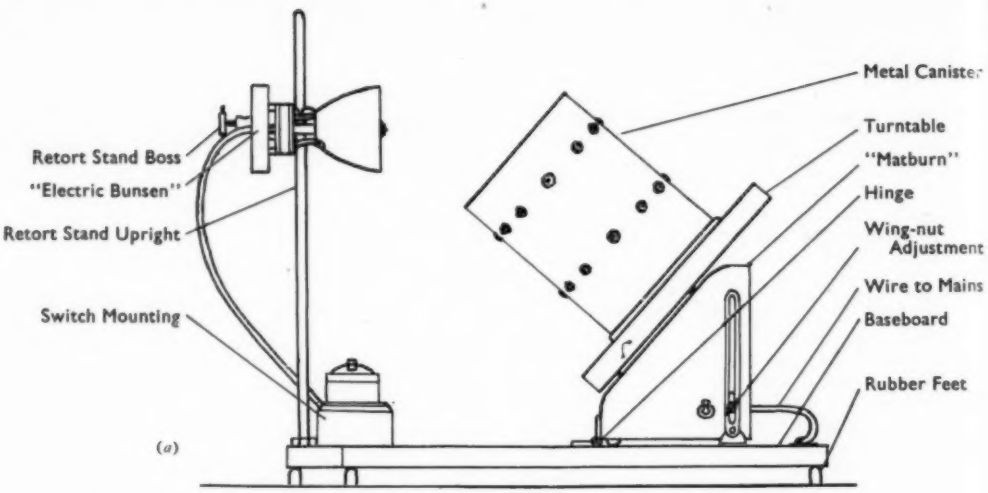
In the method of Grossmann and Grossmann (1955) for estimating plasma protein-bound iodine, the proteins are precipitated with zinc hydroxide, mixed with alkali, and dried at 95° C. overnight. This has two disadvantages: drying is slow and zinc ions have an adverse effect on the catalytic coefficient of iodide (Acland, 1957). To avoid these disadvantages Halkerston (1956), following Zieve, Vogel, and Schultz (1955) and others, used "de-acidite FF" ("permutit") to absorb inorganic iodine from the plasma and then dried the plasma with alkali. This had still the disadvantage of a slow drying process, giving a hard, thick mass on the bottom of the tube which was difficult and tedious to leach. To overcome these difficulties, it was suggested that rotating the tubes under some source of heat, such as infra-red lamps, would cause the contents to spread in a thin layer up the walls of the tube, so that drying and leaching would be facilitated.

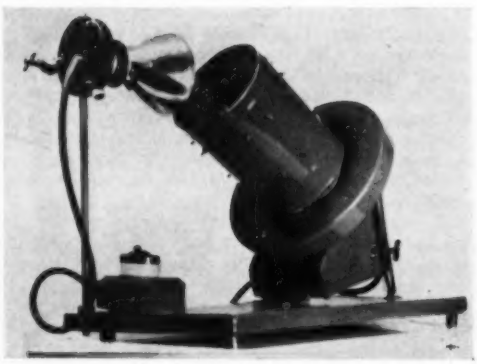
The apparatus shown in the figures was made for this purpose. On a wooden base, 50 cm. × 20 cm., a "matburn" (blood bottle) rotary mixer is attached by a hinge at the front, and a slotted brass strip and wing nut at the rear. The tilt of the turntable is thus adjustable, from 45° from the horizontal (the normal angle) to vertical. The bottle clips are removed from the turntable and to the latter is bolted an asbestos mat and to the mat a metal canister of bright tinned steel, 18 cm. × 12 cm. diameter. Two rows of ½ in. Terry clips are bolted on the inside of this container to hold 12 6 in. × ¾ in. pyrex thick-walled test tubes.

A retort stand rod bolted to one corner of the base-board carries a 340-watt electric bunsen* held in a retort stand boss by means of a bolt or a clamp rod screwed into the side of the base of the bunsen. A tumbler switch, mounted on the base-board, controls the electric supply to the bunsen.

The test tubes containing resin-treated plasma and alkali are placed in the clips inside the canister, the turntable is tilted slightly, and the machine is switched on so that the turntable and canister rotate. The heater is adjusted to point into the canister and its

*Bunray electric bunsen burner type 300 (catalogue No. 3051) supplied by Shandon Scientific Co. Ltd., 6, Cromwell Place, London, S.W.7.





(b)

FIG. 1.—(*a*) Diagram of the rotary drier, and (*b*) photograph of the assembly.

heater is switched on. Care must be exercised in the first stages of the drying process, for if the tubes are tilted too much when their contents are still very fluid, losses may occur owing to overheating and consequent spattering. As drying progresses, however, the canister can be brought into a position approaching the horizontal, in which position it will present the tubes more closely to the heater.

In use over several months the apparatus has proved very satisfactory. The plasma and alkali mixture in the tubes is usually spread to a distance of 3 to 4 cm. up the walls of the tubes, and drying is complete in four hours. Leaching after incineration is very easy, entailing only a little scraping with a glass rod, if the acid is allowed to stand in the tubes for a short time.

Some difficulty was at first experienced in the lubrication of the turntable spindle. It was found that silicone fluid D.C.550 applied occasionally was satisfactory and withstood the heating far better than a light-grade mineral oil.

The outside of the canister should preferably be kept bright to reduce heat loss.

My thanks are due to Dr. W. W. Kay, Group Pathologist, for helpful suggestions and to Mr. P. Shackcloth, F.I.M.L.T., for the photograph.

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Halkerston, I. D. K. (1956). Personal communication.
Zieve, L., Vogel, W., and Schultz, A. (1955). *Clin. Res. Proc.*, **3**, 120.

A Simple Anaesthetic Apparatus for Small Animals

J. H. CONNOLLY

From the Department of Microbiology, the Queen's University of Belfast

(RECEIVED FOR PUBLICATION DECEMBER 21, 1957)

This apparatus was developed for the anaesthesia of small animals, including infant and adult mice, rats, guinea-pigs, rabbits, and rhesus monkeys.

The general layout of the apparatus is shown in the diagram.

The apparatus consists of an electric pump which drives air through a two-way tap connected to two tubes in an ether container. One of the tubes reaches well below the surface of the ether, and the other ends above the surface. By a turn of the tap it is possible to blow air through the liquid ether or over its surface, giving a higher or lower concentration of ether vapour respectively which is then forced onwards through an outflow tube to a face mask. Two short pieces of tubing serve as the outflow tube and a filling tube respectively.

The pump used was an aquarium aerator of the vibrator type. Possible sparking from motor brushes was thus avoided and the risk of ether explosion eliminated. The pump has a needle valve which controls the air flow.

Rubber and glass tubing of 3/16 in. internal diameter was used throughout.

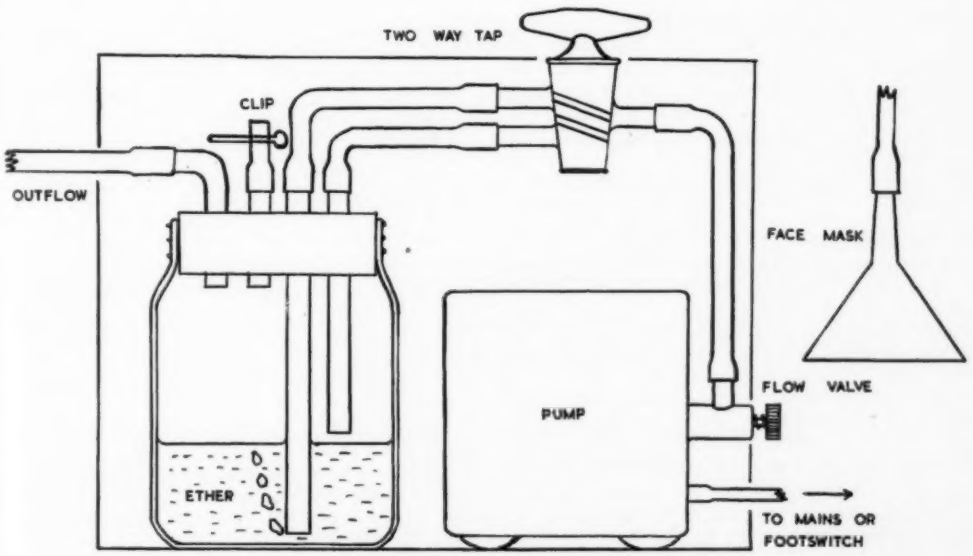
For the ether container any wide-necked flask or bottle of 250 ml. capacity will serve. A 1 lb. honey jar fitted with a rubber bung to take the four pieces of glass tubing was found to be a useful size. The two-way tap is connected to the pump and ether bottle by rubber tubing as shown in the diagram. Several feet of rubber tubing connect the outflow tube to the face mask.

The face mask is a pliable polythene filter funnel 2 in. in diameter. Alternatively a thin polythene bag 9 in. by 7 in. which covers the whole head of the animal may be used.

The apparatus is enclosed in a metal or wooden box 8 in. by 6 in. by 4 in., and the pump wired to an electric plug. For convenience the pump may be fitted with a foot switch, so that the ether vapour may be controlled when the hands are fully occupied.

A small funnel is attached to the filling tube and 50 ml. of ether poured in. When the apparatus is in use the filling tube is closed by a short piece of rubber tubing and a clip. To induce anaesthesia, the flow valve is opened to give maximum flow, and the two-way tap turned so that air is bubbling through the ether. When the animal is anaesthetized the flow is reduced and the two-way tap turned so that air is now flowing over the surface of the ether. In this manner smooth anaesthesia may be safely maintained for long periods if desired.

For the anaesthesia of mice or rats the outflow tube is placed at the bottom of the jar or box containing the animals.



To prevent evaporation of ether when the apparatus is not in use the two-way tap is turned to the neutral position and the outflow tube clipped.

This apparatus is cheap to construct and portable. In contrast to open ether techniques, both vapour flow and ether concentration are controlled, giving smoother anaesthesia and greater safety; also the animal receives the ether vapour and not the operator. There is a considerable saving in ether, since only sufficient is used to induce and maintain anaesthesia and the ether flow is switched off when not required. The apparatus is effective over a wide range of animal sizes, from infant mice up to 8 lb. monkeys.

POSTGRADUATE COURSES IN PATHOLOGY ARRANGED BY THE ASSOCIATION OF CLINICAL PATHOLOGISTS, 1958-9

Date	Subject	Institution	For further particulars apply to:—
1958			
Oct. 31 & Nov. 1	Pathology of Genito-urinary Diseases	Institute of Urology, in association with St. Peter's, St. Paul's, and St. Philip's Hospitals	Dr. Roger Pugh, Institute of Urology, 10, Henrietta Street, Covent Garden, London, W.C.1
Nov. 21 & 22	Pathology of Respiratory Diseases	Institute of Diseases of the Chest and Brompton Hospital	Dr. J. W. Clegg, Brompton Hospital, London, S.W.3
Nov. 28 & 29	The Bacteriology of Intestinal Infections	Public Health Laboratory, Colindale	The Director, Public Health Laboratory, Colindale Avenue, London, N.W.9
Dec. 5	Diseases of the Heart	Institute of Cardiology	Dr. R. E. B. Hudson, National Heart Hospital, Westmoreland Street, W.1
Dec. 13 & 14	Pathology of Diseases of the Rectum and Colon	St. Mark's Hospital	Dr. Basil Morson, St. Mark's Hospital, City Road, London, E.C.1
1959			
Jan. 9 & 10	Pathology of Diseases of Children	Institute of Child Health and Hospital for Sick Children	Dr. Martin Bodian, The Hospital for Sick Children, Gt. Ormond Street, W.C.1
Jan. 16 & 17	Obstetrical and Gynaecological Pathology and "Difficult Tumours"	Institute of Obstetrics and Gynaecology in association with the Chelsea Hospital for Women and the Royal Marsden Hospital	Dr. Magnus Haines, Institute of Obstetrics and Gynaecology, The Chelsea Hospital for Women, Dovehouse Street, S.W.3
Jan. 23 & 24	Pathology of Diseases of Bones and Joints	Institute of Orthopaedics and Royal National Orthopaedic Hospital	The Dean, Institute of Orthopaedics, Royal National Orthopaedic Hospital, 234, Great Portland Street, W.1
Jan. 30 & 31	Diseases of the Eye	Institute of Ophthalmology	Professor Norman Ashton, Institute of Ophthalmology, Judd Street, W.C.1
Feb. 6 & 7	Pathology of Diseases of the Skin	Institute of Dermatology and St. John's Hospital for Diseases of the Skin	Dr. John Oliver, St. John's Hospital for Diseases of the Skin, Lisle Street, Leicester Square, London, W.C.2
Feb. 13 & 14	Forensic Pathology	Committee of Forensic Pathology, Association of Clinical Pathology	Dr. Francis E. Camps, Department of Forensic Medicine, The London Hospital, Medical College, London, E.1
Feb. 20 & 21	Pathology of Diseases of the Ear, Nose, and Throat	Institute of Laryngology and Otology	Dr. I. Friedmann, Institute of Laryngology and Otology, 330/332, Gray's Inn Road, W.C.1
Mar. 6 & 7	Pathology of Diseases of the Nervous System	Institute of Neurology in association with the National Hospital, Queen Square, and the Maida Vale Hospital, London	Dr. John Cumings, The National Hospital for Nervous Diseases, Queen Square, London, W.C.1

Note: These "week-end" courses in pathology are intended primarily for trained pathologists of consultant or S.H.M.O. status. In exceptional circumstances pathologists of senior registrar grade or other doctors may be accepted, but only if there are vacancies. Since accommodation is limited early application is desirable. A small fee will be charged at each course and this will be payable on registration.

Note that the course on diseases of the heart is for one day only, December 5. The course on January 16 and 17 will be at the Chelsea Hospital for Women on the Friday and at the Royal Marsden Hospital on the Saturday. Dr. N. F. C. Gowing is organizing the symposium on "difficult tumours" on Saturday, January 17, and those who have registered for this course may send slides to Dr. Gowing at the Royal Marsden Hospital, provided they arrive at least one week before the course.

SYMPOSIUM ON HOSPITAL COCCAL INFECTIONS

The papers on hospital coccal infections which formed a symposium arranged by the Association of Clinical Pathologists and the Medical Research Council Committee on Cross Infection in Hospital have been edited by R. E. O. Williams and R. A. Shooter. The booklet (price 3s. 6d.) may be obtained from the **Swan Press Ltd., Eakers Row, Farringdon Road, London, E.C.1.**

J. clin. Path. (1958), **11**, 371.

ASSOCIATION OF CLINICAL PATHOLOGISTS: 60th GENERAL MEETING

The 60th general meeting of the Association of Clinical Pathologists was held in Dublin. Summaries of the scientific papers follow.

A. B. RAPER described 30 cases in which there were discrete, fibrotic nodules of the splenic pulp. Such lesions had been considered very rare, and had generally been regarded as hamartomata. The present examples had been collected in routine necropsies on Africans in Uganda, where amongst male subjects they affected over 5%, mostly young adults. Such a frequency demanded a revision of the accepted diagnosis, and a review of as many as 30 cases allowed a picture of the pathogenesis to be constructed. The essential lesion was proliferation of the reticular cells of the splenic cords. This gave way to hyalinization which, however, was not uniformly distributed, being minimal on the venous side of the (anatomical) splenic lobules, where some intact pulp might remain. The amount of surviving pulp varied, so that some lesions became wholly sclerotic and others showed sclerotic bands enclosing islands of pulp. To the naked eye, the nodules appeared as slightly raised bosses, usually on the anterior border, or as firmish spheres within the spleen; on section they were clearly demarcated, but without a true capsule, and they showed interlacing bands of greyish fibrous tissue enclosing islands of dark firm tissue. In discussing aetiology, the speaker gave reasons against regarding these lesions as hamartomata, or the result of parasitic (and in particular of filarial) infestation. Nor were they intralienal splenunculi or implants. Trauma, however, if it left the reticular framework intact, could initiate these, or very similar lesions, and the high frequency of splenic trauma in young African males was noted. But not all cases could be so explained, and it remained a possibility that the lesions resulted from the action of an unknown but specific agent capable of exciting fibroblastic proliferation in much the same manner as in Kaposi's sarcoma, which was not uncommon in the same population, though not directly associated with splenic nodules.

Megaloblastic Anaemia of Pregnancy and the Puerperium

C. GILES and EILEEN M. SHUTTLEWORTH reported an attempt to assess the true incidence of megaloblastic anaemia of pregnancy and the puerperium in north Staffordshire during the 12 months ending February 28, 1958. A haemoglobin level of 9.5 g.% was chosen as the critical level below which patients were included in the study, which comprised sternal puncture, serum iron determination, and an inquiry into the medical, occupational, and dietetic history of each patient. Altogether 369 cases came under review. In 67 of these sternal puncture revealed a megaloblastic marrow. Peripheral blood counts were seldom diagnostic. All patients were treated with folic acid, which produced reticulocyte counts varying from 8 to 33% and, as a rule, a pronounced fall in the serum iron. Histamine test meals in 53 patients showed 18 cases of achlorhydria. Fat absorption was definitely impaired in three out of 11 cases and the faecal fat content exceeded 20% in seven out of 15 cases. No seasonal incidence was noted and the age distribution was much the same as in normoblastic anaemias. The disease was commoner in multiparae and there was a 9% incidence of twin pregnancy. The overall diet during pregnancy was judged adequate in only 54% of megaloblastic anaemias as compared with 82% of normoblastic anaemias and 96% of pregnant women without anaemia. Of women with megaloblastic anaemia of pregnancy, 83% had fewer than three meat meals per week. There was less variation regarding other items of diet.

Haemolytic Episodes in Pregnancy

H. B. GOODALL (Dundee) said that haemolytic anaemia is uncommon in pregnancy, but that many different types are known. He described three types.

Haemoglobinuria in severe hypertensive toxaemia was discussed in the light of a case in which the usual haematological investigations failed to reveal the cause of the haemoglobinuria. It was considered after necropsy that the free haemoglobin had probably come from the lysis of widespread hepatic haemorrhages. Intra-caval catheterization might give proof of this hypothesis.

The assessment of haemolysis in megaloblastic anaemia was on the amount of erythrophagocytosis in the peripheral blood. In three cases in which this phenomenon was particularly marked there was severe toxaemia and shock with intra-uterine death in two cases and accidental haemorrhage in the other. There was a possibility that masses of degenerating erythrophages might cause micro-embolism and, thus, shock.

Lytic episodes were described in women responding to treatment for hypochromic anaemia characterized haematologically by erythrophagocytosis and

in four of five cases by abdominal upsets of the false labour type. In one of these cases the upset was probably due to the passage of incompatible foetal red cells through a defect in the placental barrier; a large central placental lesion was found at delivery. In the others there was no gross placental damage and the infants were compatible. Though the cause of the phenomenon was not yet known, it was considered possible that a rapid increase of iron in the blood, particularly with intensive parenteral therapy, might have damaged the maternal erythrocytes and made them more susceptible to phagocytosis. Was excessive destruction of transfused blood in transfusional siderosis possibly due to a similar mechanism? Another cause of the excessive tendency to erythrophagocytosis might be the depletion by response to iron of some factor essential for the integrity of the red cell. Reasons were given for pointing to vitamin E depletion as a possibility.

The Problem of Myasthenia Gravis

A. L. WOOLF said that many curious facts relating to myasthenia gravis had been discovered in the last 50 years and the discoverer of each had been tempted to postulate his own discovery as of primary pathogenetic significance. The problem lay in the need to integrate all data into a single theory of pathogenesis. He had himself reported (Woolf, Bagnall, Bauwens, and Bickerstaff, 1956) for the first time striking changes in the intramuscular nerve endings in a case of myasthenia gravis and had now found similar changes in two out of a further three cases.* The changes consisted of sprouting of the distal extremity of the subterminal nerve fibres (which were sometimes irregularly swollen) and formation of multiple, bizarre, or unduly complex end-plates. Cholinesterase preparations showed minute deposits of cholinesterase over a wide zone around the end-plates, indicating that there were far more sprouts than were detectable with the light microscope alone. The changes resembled those seen in myositis, dystrophia myotonica, and thyrotoxic myopathy. These diseases had little in common as regards contractility of muscle, but all showed evidence of endocrine imbalance. He thought the changes in the nerve endings were the result of muscle fibre degeneration and lymphorrhages caused by this imbalance, which was also responsible for the disordered contractility.

REFERENCE

Woolf, A. L., Bagnall, H. J., Bauwens, P., and Bickerstaff, E. R. (1956). *J. Path. Bact.*, **71**, 173.

Histology of Chronic Bronchitis

W. K. TAYLOR described the microscopical appearances of biopsy material taken from the carina in 54 cases of chronic bronchitis. All cases showed an increase of goblet cells up to 100% of the epithelial cells. Transitional cell metaplasia was a very common finding (60% of cases) and about 12% showed squamous metaplasia. Carcinoma *in situ* was seen in a few cases. In more than half the sections there was some degree of thickening of the basement membrane. In the lamina propria there was often some oedema and occasionally dilated lymphatics. An increase in vascularity was frequently noted. Cellular infiltration was notably slight and in a high proportion consisted only of a few lymphocytes, Plasma cells were not infrequent, but polymorphs were rare and eosinophils were not seen. Elastic fibres were reduced in number and often absent. Fibrosis was uncommon and where present was fine and diffuse.

*All cases referred by Dr. E. R. Bickerstaff.

Cytological Observations on Carcinoma

R. A. Q. O'MEARA and R. D. JACKSON had applied standard cytological methods to fresh biopsy material containing carcinomas. The first aim had been to determine the way in which a primary carcinoma invades the tissues, using the cytological techniques as a means of orientating the growing cells. In particular, staining of the Golgi apparatus had been useful and had shown different results in man from those obtained by Ludford (1925) in animals. Squamous cell carcinomas were first examined and it was found that the arrangement of the Golgi apparatus in the normal epidermis corresponds to that found in carcinoma invading the tissues, in so far as the basal cells of the epidermis correspond to the peripheral cells of invading carcinoma, and the mature cells of the epidermis undergoing keratinization correspond to those of the central parts of the carcinoma. Thus in squamous carcinoma the tumour is maturing by mitosis and keratinization towards the centre of the growth, while the peripheral cells of the carcinoma are growing outwards into the tissues in the opposite direction.

Concentrating on the cytological features of these cells, as representing the more important part of the growth, it was found incidentally, when stained to show mitochondria, that the cells appeared to be forming fibrils. Further investigation showed that these fibrils were not artefacts, but are deposited on the outlying invading cells. All tests so far applied indicate that they are fibrin or of a fibrinous character. This point continues to be investigated. Sometimes the invading cells are overwhelmed by the deposit and they are then removed by phagocytes: for the most part they thrive and grow outwards into the deposit. The fibrils can be found in advance of the main body of the tumour, forming a coagulum in the tissue spaces, thus interfering with the nutrition of the cells of the part and providing the carcinoma cells with an advantage. It is also considered that successive fibrinous deposits on the invading carcinoma are of importance in leading to the formation of the stroma of the tumour as a result of the organization thus induced.

REFERENCE

Ludford, R. J. (1925). *Quart. J. micr. Sci.*, **69**, 27.

The Thyroid Gland After Treatment of Thyrotoxicosis by Partial Thyroidectomy or ^{131}Iodine

R. C. CURRAN, H. ECKERT, and G. M. WILSON said that methods of treating hyperthyroidism included partial thyroidectomy and incomplete destruction of the gland by ionizing radiation, using ^{131}iodine. By searching the hospital's pathological records of many years, seven cases were found in which the portion of gland left after partial thyroidectomy was available for histological examination: the interval between the surgical operation and the necropsy or biopsy ranged from one to 14½ years. For comparison, 13 glands treated by therapeutic doses of radioactive iodine were obtained: these 13 patients died in hospital at intervals of a few days to five and a half years after treatment. Full necropsies were performed.

After partial thyroidectomy, the portion of gland left shows, with the passage of time, progressively decreasing cellular hyperplasia and increasing colloid storage. In the first weeks following treatment with ^{131}iodine, the irradiated glands showed disruption of follicles, necrosis and damage to epithelial cells, oedema and early fibrosis of the stroma, and widespread vascular lesions, including thrombosis. The more chronic effects of radiation treatment include stromal fibrosis and increasing numbers of altered epithelial cells, but more striking are the microfollicular structure and the comparative lack of colloid.

The two series of glands therefore differ histologically. They also appear to differ metabolically, as judged by the results obtained after giving a tracer dose of ^{131}iodine to patients rendered euthyroid by one or other of these two methods of treatment. In the post-thyroidectomy cases, the level of protein-bound radioiodine 48 hours after the test dose is almost invariably normal, that is, less than 0.4%. This suggests a normal iodine pool in the thyroid gland. In contrast, in the irradiated series, the protein-bound radioiodine remains high on average. This suggests a reduced iodine pool in the thyroid gland, a result apparently in harmony with the histological finding of colloid lack.

Histological Types of Pulmonary Cancers in 33 Women Living in the Industrial Area of Liège

J. FIRKET and M. A. VAN LANCKER (Centre Anticancéreux près l'Université de Liège) reported that the industrial area of Liège had the highest incidence of lung cancers in Belgium (17.10 per 100,000 inhabitants per year). Among 820 histologically and cytologically proved lung cancers within the five past years 33 female patients were found, most of whom had not been submitted to the direct action of tobacco.

In an attempt to define the aetiology of these types of cancer, meteorological, climatic, and geographical factors had been investigated and the histological types of the lung cancers analysed.

The distribution of female lung cancer histological types is very similar to the distribution of all lung cancers in western Europe, even after taking into account the controversial classification of adenocarcinomas which Kreyberg includes in his group II. In group I he places epidermoid, large and small round-cell carcinomas; in group II, adenocarcinomas. adenomatosis, and bronchial glandular tumours.

Of 33 women, eight were living in country areas; only 3 among them were to be classified in group II. The data are not sufficient to draw any significant conclusions.

The main interest of Kreyberg's work is that it yields information on areas where atmospheric pollution is relatively low and lung cancer incidence has been growing more recently than in our own areas of observation.

Control of Staphylococcal Infection in Surgical Wards

W. A. GILLESPIE, G. AYLIFFE, and V. G. ALDER said that cross-infection by *Staph. aureus* in three surgical wards had been studied for two years. About 3% of all wounds were infected by *Staph. aureus* in the theatre, sometimes with serious results. Thirty-eight per cent. of all open and drained wounds were subsequently colonized by *Staph. aureus* in the wards, but often with little effect on the wounds.

Staph. aureus caused about 10% of the post-prostatectomy urinary infections, and in about 1% of all patients caused other staphylococcal complications (pneumonia, enterocolitis, parotitis, boils).

Staphylococci with multiple antibiotic resistance caused nearly all the wound and other infections in the wards and often spread from patient to patient, causing "epidemics" of ward cross-infection. They also caused almost one-third of the theatre staphylococcal infections. The chief sources of these strains were the patients' infected open lesions and noses. They rarely spread from nurses' noses. Strains which were sensitive to all antibiotics or resistant to penicillin only were common in the noses of the staff and caused most of the theatre infections, but rarely spread in the wards.

After a seven-month control period, extra precautions were introduced into one ward during the next six months. Nasal carriers were treated with neomycin-bacitracin ointment, the blankets and pillows disinfected with formalin after use; the barber's brushes, the crockery, and baths were disinfected; open wounds were sprayed with "polybactrin"; paper towels and "hibitane" hand cream were issued to nurses. There was a moderate reduction in cross-infection, of doubtful significance. Finally, in February, 1957, the disinfection of baths was improved by adding hexachlorophene to the bath-water, and nasal prophylaxis was started by supplying twice daily neomycin-bacitracin ointment to the noses of all patients from the day of admission. Following these steps the rate of cross-infection fell immediately. During the subsequent year (to February, 1958) 13% of 106 open wounds became infected by *Staph. aureus*

compared with 47% of 60 similar wounds in the control period. There was a corresponding fall in incidence of other staphylococcal complications in the experimental period, and a reduction in theatre infection which was probably partly caused by the changes in the ward.

Subsequent study showed that nasal prophylaxis by itself gave only a slight reduction in wound cross-infection, much less than when combined with other precautions.

Haemoglobinopathies in General Hospital Practice

H. M. RICE (Nottingham) described two cases of haemoglobin C disease (CC), which were discovered within one year, from a West Indian population of about 2,000. Both cases were pregnant women and both had been quite well between pregnancies. Their haemoglobin dropped in pregnancy and haemoglobin C was not detectable in the cord blood of the infants.

Within about one month after, the haemoglobin F level was noted to drop and that of haemoglobin C to rise, and at about one year haemoglobin F was not detected and haemoglobin C was about 40%.

He also described a sickle cell anaemia in a male Jamaican aged 40 years with characteristic findings in the red cells and Hb and in the electrophoretic pattern.

Phenol Reaction for Blood Sugar Estimation

T. M. HEALY (Dublin) reported on the use of this test. Simple sugars, when treated with phenol and concentrated sulphuric acid, gave an orange-yellow colour, stable for some hours (Dubois *et al.*, 1956). Experience of the test over two years had shown that the method gave accurate and consistent results. With standard sugar solutions of 121 mg.% concentration "Iabtrol" the mean of 30 readings was 116.5 mg.% (S.D. ± 3.3) and with reconstituted plasma containing 400 mg.% the mean was 398.5 mg.% ± 7.0. Compared with Folin and Wu's method the results were of comparable accuracy, but the phenol method took one-third of the time and needed less apparatus and cheaper reagents. There had been no accidents with the concentrated sulphuric acid.

The method was found suitable for routine use.

The Prophylaxis of Pulmonary Embolism

N. B. GIBBS (Royal Surrey County Hospital, Guildford, Surrey) said that he had investigated fully the relationship between venous thrombosis of the lower limbs and bed rest. Thrombosis begins in two separate and quite clearly defined sites, namely the leg and the thigh, where venous stasis is particularly liable to occur. Venous stasis is prevented in hospital patients by early ambulation and other efforts.

A simple apparatus has been designed which enables complete control of exercise of leg and thigh muscles to be effected in patients confined to bed. The "soleus ergometer" consists of a pivoted platform attached to a spring of variable tension. The platform is depressed by plantar flexion of the feet, and each full movement of the platform is recorded on a meter. The apparatus is mounted on an adjustable carriage which is clamped to the foot of the bed. The spring tension and position of the apparatus may be adjusted with regard to the height, age, and strength of the patient. An average of 30 recorded acts of plantar flexion per hour during the waking hours is thought to be adequate.

Ornithine Carbamyl Transferase in Serum

PATRICK T. MOORE (St. Kevin's Hospital, Dublin) reported that following the method of Reichert estimates were made of the enzyme ornithine carbamyl transferase (O.C.T.) in serum. Normal values range from 1 to 5 units and elevated values are observed in cases of acute liver damage.

The simple method of estimation is based on the arsenolysis of citrulline described by Krebs, Eggleston, and Knivett as follows:

(1) Incubate 1 ml. of serum for 24 hours at 37° C. with 1 ml. 0.2M dl-citrullin in 0.5M arsenate buffer at *p*H 7.1 and a further 1 ml. of serum under similar conditions but without the substrate citrulline.

(2) Stop the reaction by adding 0.2 ml. 4N perchloric acid and centrifuge down precipitate.

(3) Estimate ammonia in 1 ml. of supernatant by the Conway microdiffusion technique. Final Nesslerization is to be preferred to titration because of the range of values observed.

(4) After subtraction of the blank, results are expressed in O.C.T. units (1 unit=1 μg. NH_3—N liberated in 24 hours per ml. serum).

The importance of elevated levels of O.C.T. lies in the fact that the enzyme can only have been derived from liver where it is specifically located as one of the enzymes of the urea cycle.

The Further Investigation of Hypercalcaemia

H. G. MORGAN (Dundee) reported that few patients with hyperparathyroidism were now seen with the classical biochemical and skeletal changes. Far more show renal tract signs, often with no specific bone changes, with but slight or even intermittent hypercalcaemia, and sometimes equivocal levels of plasma phosphorus, even in the absence of renal failure.

In further support of the diagnosis, and in the differentiation of the many other possibilities, newer tests have been advocated, sometimes over-enthusiastically, and are here assessed. Strontium infusion to assess skeletal uptake rates, the ionized fraction of plasma calcium, and phosphate deprivation are all potentially helpful, but little experience is yet avail-

able. Calcium infusion can usually distinguish the normal and hyperparathyroid subjects by the changes in plasma and urinary phosphorus, but hypercalcaemia of other causes gives identical results. Estimation of the increased rates of phosphate to creatinine clearances, or, better, of the decreased tubular resorption of phosphate (T.R.P.), can help to pick out patients for operation with normal or near normal plasma phosphate levels (and normal renal function), and exclude others by normal findings. However, misinterpretation occurs, with frequent false positives, i.e., a low T.R.P., from other causes of hypercalcaemia; from the renal failure in itself common in any hypercalcaemia; and from other unrelated conditions such as cirrhosis. Occasionally false negatives occur, i.e., normal T.R.P. in hyperparathyroidism. Expression of the T.R.P. in absolute terms (mg./100 ml. of glomerular filtrate) is more useful than the usual expression as a percentage ratio.

Cortisone has not yet been shown to reduce to normal the raised plasma calcium of hyperparathyroidism, but almost always does so in sarcoidosis and vitamin D intoxication, and frequently in malignancy. A definite fall of plasma calcium to normal probably excludes hyperparathyroidism, but the converse is not true.

Accurate and thorough clinical and chemical examination is more than ever necessary in hyperparathyroidism. None of the newer tests is pathognomonic, although sometimes thought to be so, and unnecessary operations are being done because of this.

5-Hydroxytryptophan-secreting Carcinoid Tumours

M. SANDLER (Royal Free Hospital, London) described two patients with metastasizing carcinoid tumours who were excreting 5-hydroxytryptophan and greatly increased amounts of 5-hydroxytryptamine in their urine as well as 5-hydroxyindoleacetic acid. The tumour tissue was of an unusual histological pattern. It was suggested that these patients, together with three others in the literature, form a distinct clinical subgroup of 5-hydroxytryptophan-secreting carcinoid tumours. Other unusual features included atypical flushing attacks—bright red, prolonged, and of "geographical" pattern—osseous metastases, low tumour 5-hydroxytryptamine concentration, and an enormous urinary histamine output.

Phenylacetic acid, which has recently been shown to have an inhibitory action on 5-hydroxytryptophan decarboxylase *in vitro*, was administered orally to one of these patients. Serial urinary indole analyses afterwards showed a response compatible with an inhibition *in vivo* also.

The Gliadin Loading Test in Coeliac Disease

W. J. E. JESSOP and W. F. HEMMENS (Dublin) said that gliadin (350 mg./kg. body weight) is given by mouth to a fasting patient. Blood specimens are taken fasting at one, two, three, and four hours after gliadin. The glutamine concentration in each specimen is measured.

The method of Harris (1943) was adapted to the measurement of serum glutamine concentration; 1 ml. serum was added to 3 ml. 14% trichloroacetic acid and a sample of the filtrate was heated to 70° C. for 60 minutes; the glutamine was determined as ammonia with Nessler's reagent; urea does not interfere.

According to Weijers and van de Kamer (1955), a normal child shows no change in serum glutamine during the gliadin loading test; an increase of at least 50% of the fasting level is observed in wheat-sensitive patients with coeliac disease.

Two normal children were included in this series. One showed no change in serum glutamine till the fourth hour, when a 25% rise was observed, and the other showed a decreased level after two hours. Of 10 children with coeliac disease who were known to be sensitive to wheat from previous dietary experiments, five showed the 50% increase over fasting serum glutamine indicated by Weijers and van de Kamer; three showed a rather smaller increase, and the blood glutamine level was unchanged in two.

It was concluded that the gliadin loading test did confirm the presence of wheat sensitivity in the majority of cases, but that in its present state of development it is not suitable as a routine test for coeliac disease.

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Chorion Epithelioma of the Pineal with Precocious Puberty

J. H. STEPHENS, D. F. CAPPELL, and J. L. EMERY (Glasgow) reported two cases of pineal tumour associated with precocious development in boys. (1) A precociously developed boy of 4 years was admitted with headache, vomiting, and a tumour of the pineal region. This was treated by *x* rays and about four weeks later he died suddenly of intraventricular haemorrhage. At necropsy the cerebral tumour was wholly necrotic, but the lungs contained multiple haemorrhagic nodules with the structure of chorion epithelioma, from which it is inferred that the pineal growth was of this nature. (2) A boy of 10 years presented about a year before death with signs of somatic and sexual precocity with mild polyuria. After about nine months nervous symptoms developed, with diplopia, mild fits, athetoid movements, and drowsiness. Examination revealed a pineal tumour with hydrocephalus, for which a short-circuit operation was performed. Death occurred suddenly from intraventricular haemorrhage and necropsy revealed a large haemorrhagic growth replacing the pineal gland and invading the adjacent brain substance. There were no secondary growths.

Despite the known activity of such growths in secreting chorionic gonadotrophin, the development of somatic and sexual precocity is not likely to be attributable to these effects but is thought to be similar to that responsible for precocity in other pineal growths, e.g., teratomata, and in certain lesions, both tumours, and inflammatory or destructive, in the posterior part of the hypothalamus, viz., by the interruption of the inhibitory nervous pathways which control the release of hormones from the anterior pituitary.

Familial Hypoplastic Anaemia: the Fanconi Syndrome

M. G. NELSON and J. T. LEWIS (Belfast) said that the syndrome of marrow failure with congenital anomalies first described by Fanconi had been seen in many parts of the world both in familial and sporadic form. They described a family in northern Ireland in which, of eight children, three are known to be affected. One with pancytopenia and pigmentation had died of a haemorrhagic diathesis, the parents and two children are unaffected, and two children, as yet normal, are too young for any syndrome to have developed.

The onset of the disease usually occurs at about the age of 6; the clinical course is progressive and normally death takes place after a period of some four years.

The clinical, haematological, and pathological findings were presented. Its aetiology was discussed both from the hereditary and environmental points of view. No evidence to support an endocrine dysfunction or metabolic error could be found at all. Laboratory tests for these functions are normal.

No therapeutic benefit was obtained from haematinics, B group substances, steroid hormones, or erythropoietin containing plasma.

Splenectomy has been performed on two of the cases. One severe case has been much improved. He is free from symptoms and fully employed 11 years after operation. The other case submitted to splenectomy has been less benefited.

Bronchiolitis from Nitrous Fumes

A. J. N. WARRACK (Sheffield) said that nitrous fumes were produced in industry in such processes as the dipping of metals, the manufacture of toluene and cellulose, and the descaling and pickling of steel sheets after firing and rolling.

Symptoms of poisoning varied with the concentration of the gas and the duration of exposure. Low concentrations merely provoked a mild irritation of the eyes and upper respiratory tract. Strong concentrations were liable to cause severe pulmonary oedema. Some cases, however, showed a latent interval of many hours, or even days, before symptoms appeared. These took the form of dyspnoea, cyanosis, and paroxysmal cough. Secondary infection is a danger and appropriate treatment might save the patient's life.

One patient had recovered after exposure to nitrous fumes whilst dipping cadmium-lined bolts in an acid mixture. He was admitted to hospital 17 days afterwards. His symptoms had not started until seven days after exposure. He recovered with massive doses of antibiotics. Chest radiographs taken at the height of his illness showed soft irregular opacities through both lung fields which disappeared eight weeks later.

Another case ended fatally. A man carrying nitric acid in a rubber bucket noticed a leak and held the bucket over another of galvanized iron. He was enveloped in thick brown fumes for 30 seconds. He complained of no ill effects and finished his work. It was not until seven days after exposure that he sought medical attention. He was admitted semi-comatose and cyanosed to hospital two weeks after the original incident. A radiograph of the chest had an appearance similar to that of the first case. Post-mortem examination showed both bronchial trees filled with thick mucopus. The lungs were solid and on section showed generalized tiny yellow areas having the appearance of purulent bronchiolitis.

Microscopical examination showed varying degrees of obliterative bronchiolitis from simple epithelial desquamation to complete bronchiolar destruction with surrounding alveolar organization. There was presumably secondary infection of these areas and it was thus important that antibiotic therapy should be given.

Both the cases described had the typical features of the condition—a latent interval between exposure and symptoms, the typical radiological appearances of obliterative bronchiolitis, in one instance recovery with adequate treatment, and in the other classical post-mortem histological findings.

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TWO CASES OF MARROW FAILURE WITH MASSIVE MARROW PLASMOCYTOSIS SIMULATING MYELOMATOSIS

BY

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It is sometimes difficult to assess the significance of an increased proportion of plasma cells in the bone marrow, although it is generally accepted that figures greater than 2% are outside the normal range (Wintrobe, 1956). An obvious increase may occur either as a simple reactive phenomenon accompanying a variety of diseases and without known significance, or as a disease *sui generis* in myelomatosis. In general a reactive increase is relatively slight whereas in myelomatosis the increase is gross and confirmation of the diagnosis will almost certainly be obtainable radiologically or biochemically. Difficulty in assessment is commonly experienced when the increase of plasma cells in the marrow is only moderate, but it may not be generally realized that this difficulty may persist when there is a marrow plasmocytosis of 60% or 70%. Such a finding, even when accompanied by a greatly increased E.S.R. and gross hyperglobulinaemia, does not always constitute evidence of myelomatosis, for these changes may also be essentially reactive in nature. Two illustrative examples are presented.

Case Reports

Case 1.—A woman aged 61 died in the Royal Mental Hospital, Glasgow, on December 17, 1955. During the previous six years she had been treated for chronic anaemia by her own doctor with iron and injections of liver extract. These raised the haemoglobin level from 50% (7.4 g. per 100 ml.) to 90% (13.0 g. per 100 ml.), as estimated by the alkaline haematin method (100% = 14.8 g. per 100 ml.). When treatment was stopped her condition invariably relapsed. No detailed haematological examination was performed during this period, but it is known that the white cell count was within normal limits.

During the last year of her life she complained of increasing deafness for which she was treated with dry thyroid, 2 grains daily, for a period of 10 weeks. After two months of this treatment she began to suffer from mild depression followed by a period of excitement. Two weeks after the onset of these emotional disturbances she was admitted to hospital (December 3, 1955), where it was considered that she was suffering from an attack of mania possibly complicated by confusion secondary to some toxic process. Clinical examination at this time disclosed no abnormality other than anaemia.

Laboratory Findings.—Blood examination on admission gave the following results:—Hb 51.5% (7.6 g. per 100 ml.), P.C.V. 22%, M.C.H.C. 34.6%, W.B.C. 1,850 per c.mm., and reticulocytes 2% of the R.B.C. Within a few days the leucocyte count had fallen to 750 per c.mm. and blood films showed marked rouleaux formation with a severe leucopenia predominantly affecting the neutrophils; no primitive white cells or plasma cells were present, and their absence was confirmed by examination of a buffy coat preparation; platelets were in normal numbers. Smears and histological sections of the sternal marrow were examined (Cappell, Hutchison, and Smith, 1947). Although the fragments were in general rather more cellular than normal this increase in cellularity was less than was to be expected in view of the pronounced anaemia (Fig. 1); megakaryocytes were present in normal numbers; 75% of the cells were plasma cells with a few young forms showing definite nucleoli, but most were of mature type (Fig. 2); a marked reduction in the number of both red and white cell precursors was evident. The picture was that of a severe hypoplasia of the blood-forming elements with gross secondary plasmocytic infiltration. The E.S.R. was considerably raised. The serum albumin was 2.29 g. per 100 ml. and the serum globulin 3.29 g. per 100 ml., giving an albumin-globulin ratio of 0.69. Bence-Jones proteinuria was not present.

Progress.—Nine days after admission she developed acute bronchopneumonia which failed to respond to treatment with penicillin. A slow drip of 2 pints of fresh Group O blood brought no improvement, and she died five days later.

Post-mortem Findings.—There was considerable rarefaction of the bodies of the lumbar and lower thoracic vertebrae and the marrow was much paler than normal. The sternal marrow was pink; a small

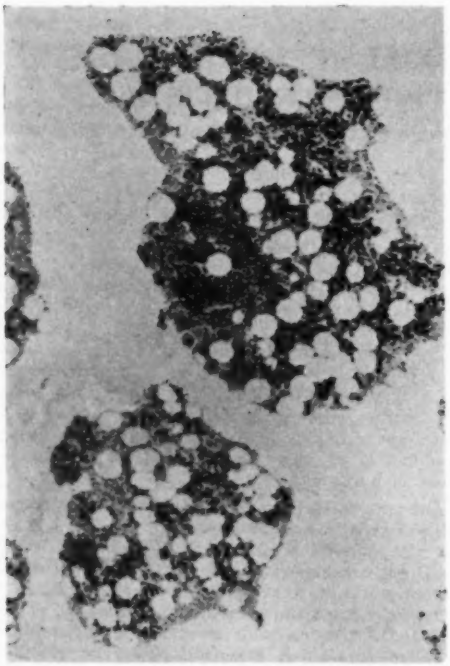

FIG. 1.—Case 1. Low-power view of the histological section of the sternal marrow biopsy showing the relatively slight increase in cellularity. Haematoxylin and eosin × 70.

amount of reddish marrow was present at the upper end of the shaft of the right femur, and the remainder of the marrow cavity was filled with yellow fatty marrow in which there were occasional small red foci. No nodules of tumour were evident in any of these marrow spaces. Microscopical examination of marrow from the sternum, vertebrae, and upper end of the femur revealed an almost complete absence of haemopoietic elements, the great majority of the cells present in the generally fatty marrow being plasma cells. The lungs were very oedematous; the left lower lobe was intensely congested and covered by fibrin; vomitus was present in the air passages. Microscopy showed an acute haemorrhagic bronchopneumonia characterized by almost complete absence of inflammatory cells. Lentil seeds in many of the bronchioles (Head, 1956), and considerable digestion of the lung parenchyma, provided definite evidence of aspiration of vomitus (Fig. 3). The liver (1,850 g.) showed centrilobular congestion and necrosis. The spleen (360 g.) contained an unusually large number of plasma cells. The thyroid gland was of normal size, and although microscopy showed inactivity the appearances were not those seen in myxoedema.

Case 2.—A man aged 72 was admitted to Heathfield Hospital, Ayr, on September 27, 1956, with a pustular, crusted eruption over the lips and lower jaw, apparently a sensitivity reaction following treatment for parotitis with sulphonamides and penicillin.

Clinical Findings.—He was pale, obese, and slightly icteric. No lymph nodes were palpable in the axillae or groins, but the cervical lymph nodes could not be adequately examined because of the skin condition. The tongue was moist and clean, but the fauces were not seen as the mouth could not be fully opened. There was a tachycardia of 114 per minute, with a respiratory rate of 26 per minute and a blood pressure of 100/50 mm. Hg. The temperature at this time was normal, though an intermittent pyrexia of up to 101° F. appeared later. There was marked sacral and slight ankle oedema and some overfilling of the neck veins was noted. The spleen was not enlarged. No other abnormality was found on clinical examination.

Laboratory Findings.—The haemoglobin was 21% (3.1 g. per 100 ml.), the P.C.V. 10%, the M.C.H.C. 31%, the reticulocytes 0.25% of the red cells, and the W.B.C. 800 per c.mm. The film revealed marked rouleaux formation and a moderate degree of anisocytosis; platelets were present in normal numbers; neutrophil polymorphs formed only 9% (72 per

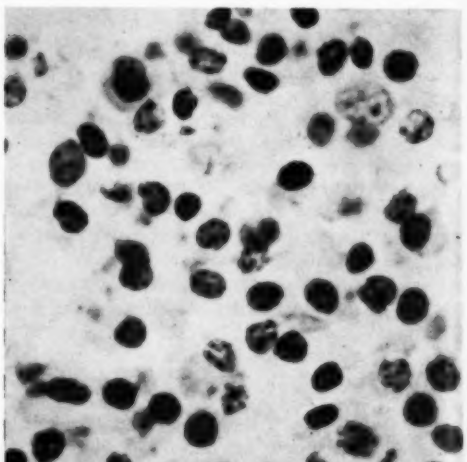

FIG. 2.—Case 1. High-power view of marrow section showing numerous plasma cells and marked reduction in normal marrow elements. Haematoxylin and eosin × 1,140.

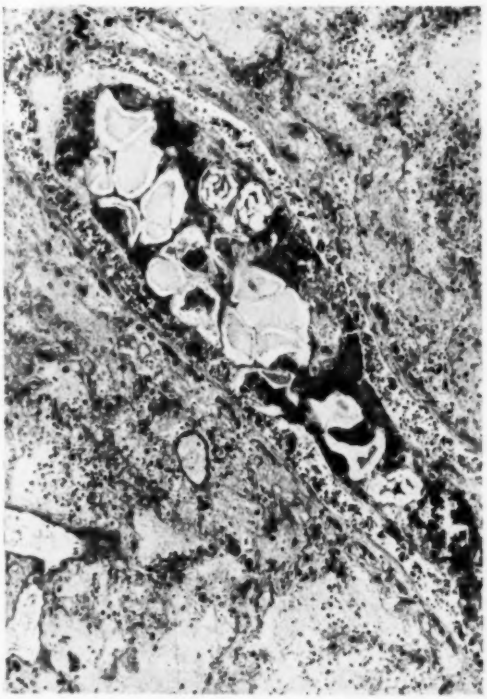

FIG. 3.—Case 1. Vomitus, including lentil seeds, in bronchiole. Note sparse cellular infiltration in adjacent consolidated lung. Haematoxylin and eosin × 72.

c.mm.) of the total white cells. Smears of the sternal marrow showed a pronounced infiltration by plasma cells (55%) and to a lesser extent by lymphocytes (Fig. 4). The plasma cells were for the most part of mature type, but a small proportion appeared atypical and occasional vacuolated binucleate forms were noted. Normal marrow elements were markedly reduced in numbers: apart from a few rather small atypical myeloblasts, occasional neutrophil polymorphs and metamyelocytes were the only myeloid elements present; the erythroid series was represented by small numbers of normoblasts at varying stages of development, the majority being late fully haemoglobinized forms; megakaryocytes were present, but in small numbers. Histological examination of the marrow fragments showed that they were of normal cellularity, but in view of the severe anaemia and the high proportion of plasmocytes this was taken to indicate hypoplasia of the normal marrow elements.

The serum proteins were 6.9 g. per 100 ml. (albumin 3.2 g. per 100 ml. and globulin 3.7 g. per 100 ml.), with an albumin-globulin ratio of 0.87. Electrophoresis showed an increase in the gamma globulin fraction. The serum calcium, phosphorus, and alkaline phosphatase levels were normal. The serum bilirubin was 7.4 mg. per 100 ml. and serum uric acid 3.5 mg. per 100 ml. Bence-Jones proteinuria was absent.

Radiography of the skull and long bones showed no evidence of myelomatosis.

Course and Treatment.—For the first two weeks the patient's condition remained precarious and he was disorientated and delirious. Shortly after admission he was given a transfusion of 4 pints of fresh blood which raised the haemoglobin to 47% (6.9 g. per 100 ml.). Intravenous terramycin and intramuscular penicillin therapy was instituted and he was also given pentose nucleotide, 10 ml. intramuscularly twice daily. Urinary retention had to be relieved by an indwelling catheter, and an infection of the urinary tract which followed proved most resistant to antibiotic therapy including streptomycin and chloramphenicol. On the twelfth day after admission 2 further pints of whole blood were transfused, and on the fourteenth day the haemoglobin was 61% (9.0 g. per 100 ml.). During this two-week period the white count varied from 500 to 1,200/c.mm. with up to 20% granulocytes; on several occasions a few plasma cells were seen in the

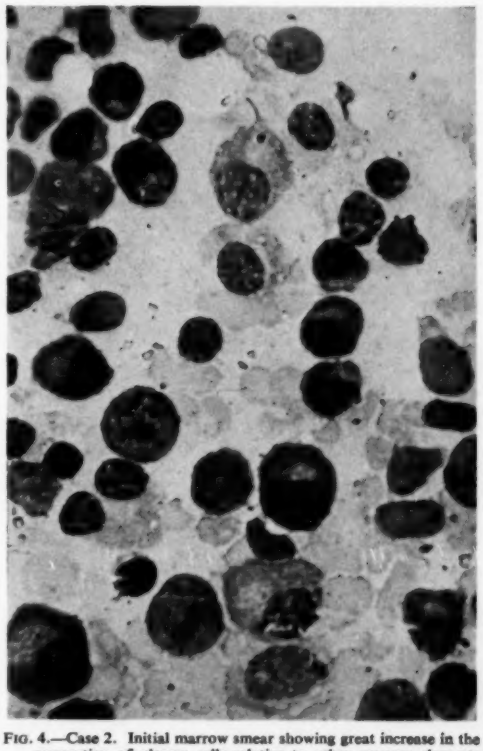

FIG. 4.—Case 2. Initial marrow smear showing great increase in the proportion of plasma cells relative to other marrow elements which are considerably diminished. Leishman × 800.

peripheral blood; reticulocytes were constantly less than 1% of the red cells. On the fifteenth day sternal puncture was repeated. The findings were essentially unchanged except for a slight fall in the number of plasma cells and a corresponding rise in the number of erythroid and myeloid elements.

From this date the patient's condition began to improve slowly but progressively. The white cell count slowly rose and this was accompanied by a gradual increase in the proportion of granulocytes and by a low reticulocytosis which reached a maximum of 4.5% on the 26th day. By this time the white count had reached 5,200/c.mm. with 78% granulocytes; there was a prominent shift to the left and the frequent juvenile forms, metamyelocytes, and even myelocytes, indicated considerable marrow activity. Occasional normoblasts were also noted in the blood film. A further marrow puncture showed a continuing increase in the number of normal marrow elements, with a diminishing plasma cell content.

The rise in the white count continued: on the 33rd day it reached 15,300/c.mm. and for the remainder of the patient's stay in hospital it did not fall below 11,000/c.mm. The haemoglobin responded more slowly and several blood transfusions were required at short intervals to maintain it at from 54 to 61% (8.0 to 9.0 g. per 100 ml.). These blood findings were considered to be a result of the urinary tract infection, for a raised blood urea of up to 130 mg. per 100 ml. indicated some impairment of renal function.

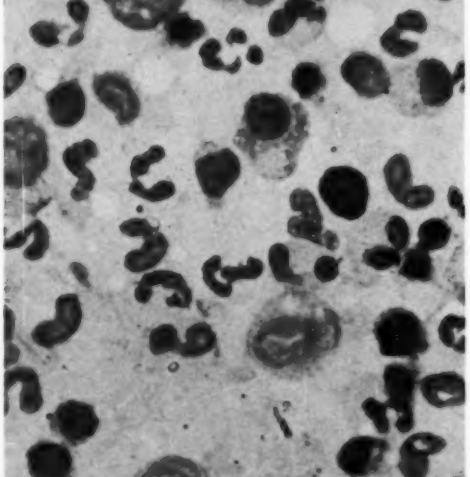

FIG. 5.—Case 2. Marrow smear on 55th day of illness showing return to normal with only scanty plasma cells and numerous segmented neutrophils. Leishman × 800.

On the 55th day the bone marrow had almost returned to normal; it was of average cellularity and contained only 2% plasma cells; no abnormality was noted in the myeloid series apart from a slight eosinophilia, and segmented neutrophils were numerous (Fig. 5). The serum protein pattern also showed a progressive improvement, the albumin-globulin ratio returning to normal with a gradual fall in the gamma globulin.

On the 77th day the patient was transferred to Ballochmyle Hospital, Mauchline, for plastic repair of a skin defect of one thigh following a large abscess which had developed at the site of the pentose nucleotide injections. During the next two months further blood transfusions were given on two occasions to hasten the return of the haemoglobin to normal. Renal function gradually improved with an accompanying fall in the blood urea. When discharged on February 15, 1957, with his leg soundly healed, peripheral blood and blood urea concentrations were normal and electrophoresis of serum gave a normal protein pattern. He was still well, with normal laboratory findings, when last seen in April, 1957.

Discussion

Both these patients presented with severe anaemia and granulocytopenia amounting almost to agranulocytosis. When further investigation showed a massive infiltration of the bone marrow by plasma cells, rouleaux formation, a high E.S.R., and hyperglobulinaemia, a diagnosis of myelomatosis was seriously considered. Anaemia is common in this condition and may indeed be the presenting feature; also leucopenia was present in 40% of the cases reviewed by Snapper, Turner, and Moscovitz (1953). While the plasma cell infiltration in the marrow smears from these patients was heavy, the great majority were of normal mature type, and although there were a few atypical and immature cells these were not more numerous than is commonly found in secondary plasmocytosis. Although some authorities hold that the so-called myeloma cell has characteristics which distinguish it from the plasma cell (Wintrobe, 1956; Snapper *et al.*, 1953) histological examination of the marrow particles had been found here to be more helpful and reliable in resolving the diagnostic problem. In myelomatosis there is a spurious appearance of hyperplasia, but the replacement of the fat is actually due to infiltration by plasma cells which occur in clumps and sheets while the usual

marrow elements are not obviously increased in amount. In secondary plasmocytosis, on the other hand, the overall cellularity is not grossly increased, for plasma cells have merely taken the place of the normal marrow elements as in our own two cases. In addition, Bence-Jones protein was absent from the urine of both patients and there were no skeletal changes at necropsy in Case 1 nor radiologically in Case 2. The normal uric acid level in Case 2 provided further evidence against myelomatosis, for the serum uric acid is almost constantly raised in myelomatosis whereas in non-myeloma cases it is usually within normal limits if renal function is unimpaired. Finally, in Case 2, conclusive evidence for the benign nature of the infiltration was afforded by the progressive disappearance of plasma cells as recovery took place.

Secondary plasmocytosis, sometimes accompanied by a plasma cell infiltration of other organs, has been recorded in a great variety of apparently unrelated disorders including acute rheumatism, rheumatoid arthritis, liver disease, Hodgkin's disease, and carcinoma (see Clark and Muirhead, 1954). The proportion of plasma cells is often considerable (Fadem, 1952), the highest proportions hitherto recorded being 55% in a patient who was hypersensitive to sulphonamides (Wolf and Worken, 1954), and 60.2% in a patient with agranulocytosis of aplastic type (Paris and Bakke, 1956). In one of the cases described here there was an even greater reactive plasmocytosis than in either of these.

An altered serum protein pattern and a raised E.S.R. usually accompany any considerable degree of secondary marrow plasmocytosis (Clark and Muirhead, 1954), and, conversely, it has been found that patients with an altered pattern usually have some marrow plasmocytosis (Bing and Plum, 1937). Some authorities speak of a fairly close correlation between the degree of alteration in the pattern and the extent of the marrow plasmocytosis (Bing and Plum, 1937; Jarrold and Vilter, 1949; Clark and Muirhead, 1954), but others have failed to confirm this (Berlin, Wallace, and Meyer, 1950), probably because the marrow plasmocytosis does not necessarily reflect the infiltration in other organs such as the spleen (Paris and Bakke, 1956). The usual alteration in plasma proteins is an increase in the gamma globulin, but it may not be discerned unless electrophoresis is carried out, or when detected it may be accepted as evidence of myelomatosis.

The occurrence of serum protein changes in secondary plasmocytosis emphasizes the close relation between plasma cells, the serum protein, and antibodies (Bjørneboe, Gormsen, and Lundquist, 1947; Fagraeus, 1948). It is now almost universally accepted that plasma cells, or more probably their precursors, are the source of antibodies which constitute a significant fraction of the gamma globulins. This hypothesis clarifies the significance of marrow plasmocytosis and its accompanying serum protein change in a great variety of conditions. An infective or allergic aetiology has been postulated for many of the diseases associated with marrow plasmocytosis, and in these the plasmocytosis may represent a response to some bacterial or other antigen while the altered serum protein pattern reflects the output of antibodies by the plasma cells. It is an interesting speculation whether the more marked degrees of plasmocytosis seen in agranulocytosis and in hypoplasia or aplasia of the marrow may indicate an excessive production of antibodies in an attempt to compensate for the lack of protective granulocytes.

Summary

Two cases are reported in which marrow hypoplasia was associated with a gross secondary infiltration of the marrow by plasma cells and an alteration in the serum protein pattern.

These cases are used to exemplify the difficulty which may arise in differentiating between reactive plasmocytosis and myelomatosis, and the value of the histological examination of marrow fragments in such cases is emphasized.

The relationship between marrow plasmocytosis and changes in the serum protein pattern is discussed.

We wish to thank Dr. Angus MacNiven, Physician Superintendent of the Glasgow Royal Mental Hospital, and Dr. R. Hill, consultant physician, Heathfield Hospital, Ayr, for access to the clinical records of Cases 1 and 2 respectively. The photomicrographs were taken by Mr. G. Kerr.

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THE SYSTEMIC LESIONS OF WHIPPLE'S DISEASE

BY

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In the 50 years since the first case report by Whipple (1907), the British contribution to the subject of "intestinal lipodystrophy" consists of five communications, namely, those of Glynn and Rosenheim (1938) and of Vaux (1943), in neither of which the condition was recognized, and the more recent reports of Avery Jones and Paulley (1949), Christie and Galton (1952), and Paulley (1952). All these papers are clinical in aspect, and due attention has been paid in them, and also in annotations in both the *Lancet* (1953) and the *British Medical Journal* (1955 and 1957), to the many bizarre features which constitute the syndrome. This, in its classical form, is a disease of insidious onset, affecting mainly male subjects of middle age. It is frequently associated with a multiple arthritis, severe steatorrhoea, and steady decline ending in death.

In contrast to this small number, there have been 28 case reports from America since 1950, of which 10 were diagnosed before death by laparotomy and mesenteric node biopsy, and five suspected on clinical grounds alone. Recognition of the disease during life has of late assumed added importance because of the emphasis on treatment in recent American articles (Jones, Benson, and Roque, 1953; Lepore, 1954; Wang, Janowitz, and Adlersberg, 1956) and also because the publication during the same period of several small series of cases (Hendrix, Black-Schaffer, Withers, and Handler, 1950; Russo, 1952; Casselman, Macrae, and Simmons, 1954; Puite and Tesluk, 1955) suggests that the condition may be more common than has hitherto been suspected.

The Present Study

The present paper is concerned with seven cases of the condition occurring in England: the main aetiological and clinical features of this group are summarized in Table I. The main pathological interest has always centred upon the jejunal mucosa and mesenteric lymph nodes, where the presence of macrophages containing Schiff-positive material is invariable, and essential for diagnosis (Figs. 1, 2a, and 2b). In addition, however, more widespread lesions are usually present, as can be seen from Table II, which summarizes the necropsy findings in the 60 adequately documented cases previously reported, and the present series.

TABLE I
THE AETIOLOGY AND SYMPTOMS OF SEVEN CASES OF WHIPPLE'S DISEASE

Case	1	2	3	4	5	6	7
Sex	M	M	M	M	M	F	M
Age at onset of symptoms (yr.)	32	48	48	39	51	23	44
Age on admission to hospital	37	48	63	47	54	46	48
Duration in hospital (mth.)	3	14	3½	18	6	5	3

The Prodromal Period			Period in Hospital	
Symptoms	Average Duration		Clinical Features	No. of Cases
	0–5 yr.	5–10 yr.		
Asthenia	4	Nil	Asthenia	4
Arthritis	2	2	Emaciation	5
Abdominal symptoms:			Hypotension	6
			Pigmentation	5
Distension	2	1	Abdominal signs:	
Constipation	3	Nil	Distension	6
Diarrhoea	1	1	Mass	1
Pain	3	1	Pain or tenderness	3
Loss of weight:			Ankle oedema	3
Mild	2	Nil	Lymphadenopathy:	
Moderate	1	,,	Localized	2
Severe	2	,,	Generalized	3
Cough:			Purpura	1
Productive	2	,,	Fever	2
Non-productive	Nil	2	Heart murmurs	1
			"Sudden" death	1

Thus, fluid and adhesions in the serous cavities are common, as are thickening and "sugar-icing" of the splenic and liver capsules. Whereas mesenteric adenopathy is invariable, lymph node enlargement elsewhere is frequently recorded, particularly in the thoracic and para-aortic regions, as well as at the periphery, where localized or generalized adenopathy is present in over half the published case reports. As part of the general investigation of the condition, peripheral lymph-node biopsy has been performed on several occasions, and a number of observers (Upton, 1952; Russo, Case 2, 1952; Fisher and Whitman,

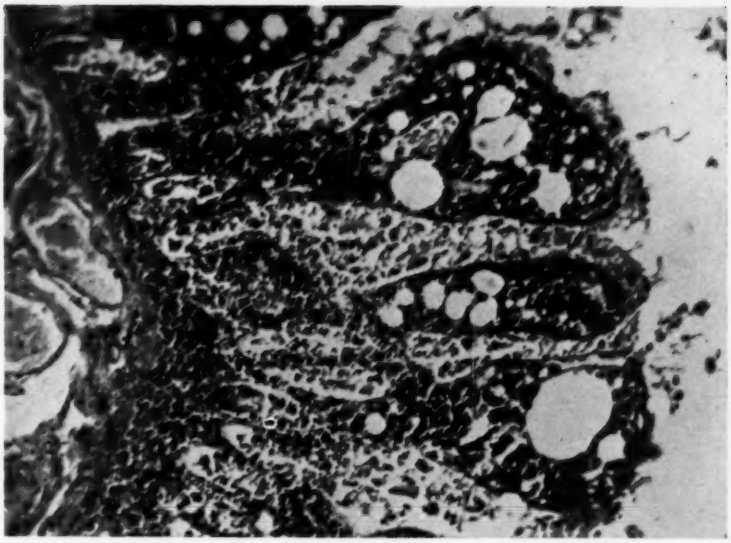

FIG. 1.—Section of the jejunal mucosa (×75) shows the club-shaped villi containing fat-filled spaces which are surrounded by large numbers of macrophages which are filled with densely staining Schiff-positive granules.

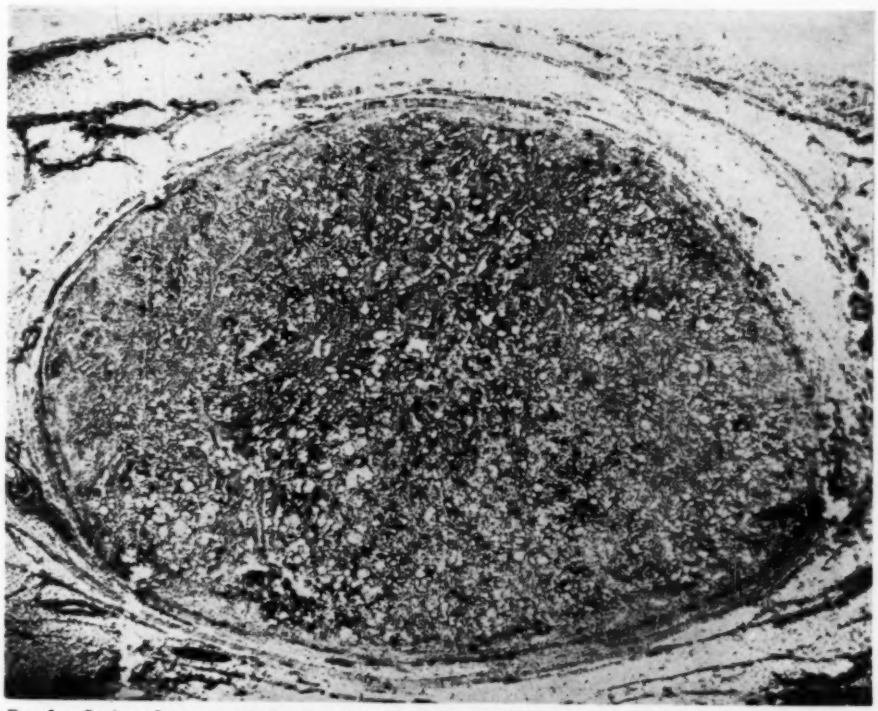

FIG. 2*a*.—Section of a mesenteric lymph-node (×16) shows the characteristic "honeycomb" appearance, the spaces containing many large fat droplets.

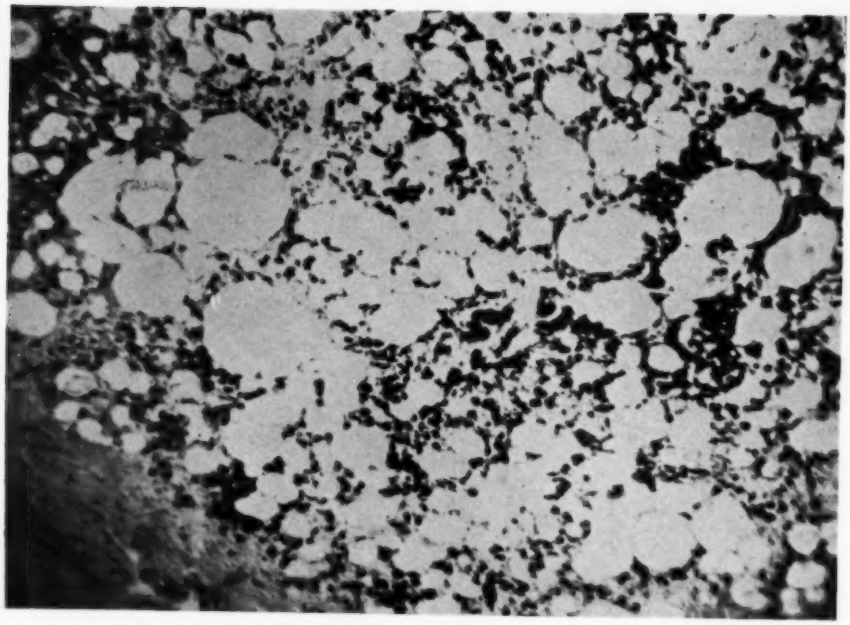

FIG. 2*b*.—A high-power view (×75) shows the fat spaces interspersed with dense aggregations of Schiff-positive macrophages.

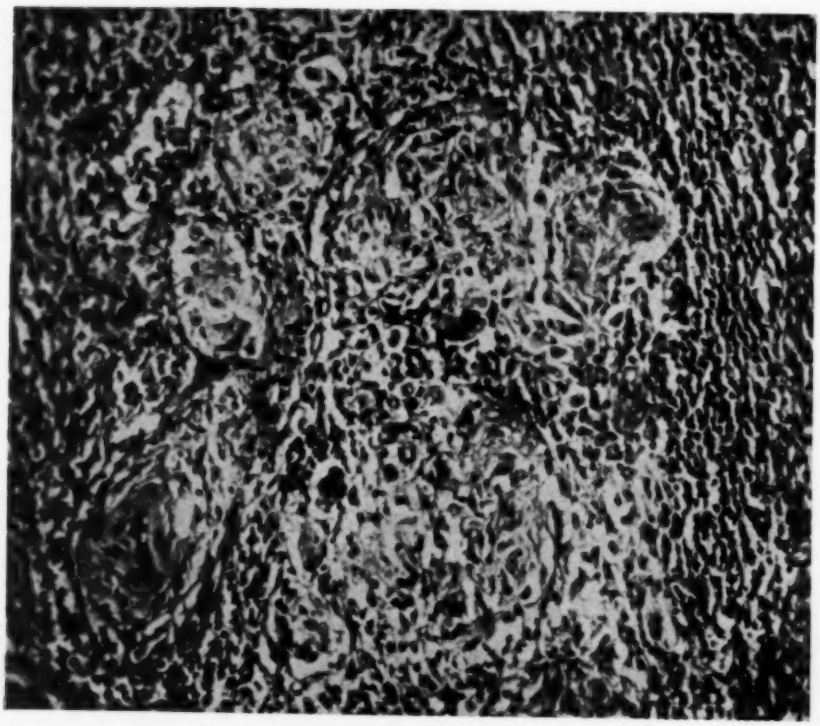

FIG. 3.—Section of a peripheral lymph node shows an indefinite "sarcoid-like" lesion (Case 5) composed of epithelioid and giant-cell aggregations, with a few Schiff-positive macrophages arranged singly.

TABLE II

MAIN GROSS AND MICROSCOPIC FINDINGS AT NECROPSY IN 67 CASES OF WHIPPLE'S DISEASE

Finding	60 Reported Cases	Present Series of 7 Cases	%
Mesenteric adenopathy (with Schiff-positive macrophages)	56	7	95
Similar macrophages in jejunal mucosa	54	7	92
Marked loss of subcutaneous fat	50	6	84
Skin pigmentation	36	5	62
Petechial haemorrhages in skin	24	3	41
Peripheral lymphadenopathy:			
Localized	16	2	27
Generalized	7	3	15
Adenopathy elsewhere, e.g., thorax, para-aortic regions	18	6	36
Involvement of serous cavities:			
Pericardial fluid	6	2	12
,, adhesions	21	6	40
Subepicardial macrophages (Schiff-positive)	1		
Pleural fluid	10	3	20
,, adhesions	17	3	30
Subpleural macrophages (Schiff-positive)	1		
Peritoneal fluid	10	3	20
,, adhesions	7	1	12
Thickening and "sugar-icing" of:			
Splenic capsule	13	3	24
Liver ,,	9	3	18
Endocardial vegetations:			
Aortic valve	8	2	15
Mitral	8	2	15
Tricuspid valve	7	2	14
Pulmonary ,,	Nil	1	
Schiff-positive aggregations in vegetation	,,	1	
Histological foci of macrophages elsewhere	1	1	
Schiff-positive macrophages in peripheral lymph nodes	3	1	6

1954) have recorded in very similar phraseology the finding of an "indefinite sarcoid-like picture," a presenting feature which was also found in Case 5 of the present series (Fig. 3).

The observation by Fisher and Whitman (1954) that the presenting histological picture was "too non-specific to be of diagnostic value" was to be later refuted by Puite and Tesluk (1955), who found small clusters of the characteristic Schiff-positive macrophages in the substance of the sarcoid-like follicles. To explain this finding, which was also confirmed in Case 5 of the present series (Fig. 3), these authors postulated that "some of the material must travel by way of the blood stream"; and there is additional support, both clinical and pathological, for the idea that the material is disseminated periodically by this route.

Thus, in Case 1 of the present series, endocardial vegetations were responsible for destruction of the aortic valve and the late development of classical aortic regurgitation; and vegetations, affecting equally the aortic, mitral, and tricuspid valves, are present in over one-third of the case reports. Except in the case of Upton (1952), these vegetations do not appear previously to have been examined microscopically, but in Case 7 of the present series small aggregations of Schiff-positive material were clearly demonstrable in the substance of the vegetation (Figs. 5 and 6), indicating not only spread by the blood stream but the possibility that this material had caused a predisposing valvulitis.

In similar manner, it is probable that the widespread lesions represent an inflammatory or "allergic" reaction to small amounts of the material, an idea which would be in keeping with the features of the prodromal period, which is characterized by acute exacerbations of protean symptoms alternating with long periods of freedom. The characteristic macrophages have been demonstrated in widespread sites—liver, spleen, retroperitoneal tissues—by Upton (1952) and also in Case 5 of the present series, where they were observed in the subpleural and subepicardial regions as well as in the retroperitoneal tissues. Careful search is necessary to demonstrate them, and they may be present more frequently than suspected.

TABLE III

MAIN HISTOCHEMICAL FINDINGS IN MESENTERIC LYMPH NODES AND JEJUNAL MUCOSA IN WHIPPLE'S DISEASE

Substance Tested	Method	Result
Fat stains (frozen sections)	Oil red O	Positive
	Sudan black B	,,
	Sudan III and IV	,,
	Nile blue sulphate	,,
	Schultz's method for cholesterol	,,
Carbohydrate	Periodic-acid-Schiff (Hotchkiss, 1948)	Positive
	(*a*) P.A.S. after acetylation;	(*a*) Positive reaction (Schiff neg.)
	(*b*) ,, after subsequent deacetylation (McManus and Cason, 1950)	(*b*) Positive reaction (Schiff positive)
	P.A.S. after diastase	No change
	,, ,, hyaluronidase	Some diminution in colour intensity
Protein	Xanthoprotein reaction (Serra, 1946)	Negative (tryptophane)
	Millon reaction (Pearse, 1953)	Negative (tyrosine)
	Sakaguchi reaction (Baker's modification)	Moderately positive (arginine)
Miscellaneous	Metachromatic stains:	
	1% Toluidine blue	Positive
	1% Thionin	,,
	1% Methyl violet	,,
	Dialysed iron (Hale, 1946)	,,
	Methylene blue extinction (range *p*H 1.9–9.5)	Positive to level *p*H 3·6
	Alcian blue (mucin)	Positive
	Mucihaematin (Laskey, 1950)	Negative
	Perls's reaction (Fe)	,,

The stains for fat refer only to the enlarged cystic spaces in the mucosa and mesenteric lymph nodes.

FIG. 4

FIG. 4.—(×30). A small vegetation from the pulmonary valve (Case 7) shows the incorporation of small groups of Schiff-positive macrophages.

FIG. 5.—A high-power (×280) view of the area showing Schiff-positive granules.

Histochemical and Experimental Considerations

Since the investigations of Black-Schaffer (1949), attention has been increasingly focused upon the macrophage which is characteristic of the disease. Its properties have been studied by several authors (Upton, 1952; Fisher and Whitman, 1954) and its histochemical characteristics have been subsequently investigated most carefully by Casselman *et al.* (1954). The main investigations on this material in the present study are summarized in Table III, from which the same general conclusion is drawn as in previous studies, that the material is a mucopolysaccharide. The main differences from the findings of Casselman *et al.* (1954) were that the tests for protein were only very slightly positive; that the methylene blue extinction test was positive down to a level of *p*H 3.6, which was

FIG. 5

also reported by Jones *et al.* (quoted by Casselman); and that some impression appeared to be made upon the material after incubation with hyaluronidase.

Although the stored material has been characterized on several occasions, there has been no attempt to reproduce the characteristic histiocyte in the experimental animal, or to study the systemic effects of circulating mucopolysaccharide. The only authors whose experimental observations are quoted in connexion with Whipple's disease are Hass (1938) and Stryker (1941), the former having studied the tissue reactions to natural oils and their derivatives, while the latter was concerned with the tracing and disposal of large quantities of liquid paraffin after ingestion. Both authors were working at a time when the basis of the condition was thought to be a disturbance of fat metabolism, but the lesions observed bear no more than a superficial resemblance to the characteristic appearances of Whipple's disease.

With the advent and use of specially refined "dextran," the effects of circulating polysaccharides have been investigated by a number of authors (Goldenberg, Crane, and Popper, 1947; Engstrand and Åberg, 1950; Persson, 1952; Friberg, Graf, and Åberg, 1953; and Wolman, 1956), but an examination of the tissue reactions to the mucopolysaccharide stored in Whipple's disease must await its specific identification. However, due to the work of Meyer (1938) and Stacey (1943, 1946), a number of substitutes are available. The former included only hexosamine-containing compounds, but Stacey included many other complex carbohydrates, which he divided into four main groups, viz., (*a*) containing hexosamine and uronic acid; (*b*) containing no uronic acid; (*c*) containing no hexosamine; and (*d*) without hexosamine or uronic acid residues. The first of these groups includes such substances as hyaluronic acid, heparin, chrondroitin sulphate and mucoitin sulphate (from gastric mucin), and since this latter material was readily available it was chosen for further experimental observation.

Materials and Methods

The pilot experiments were carried out on five rabbits and six mice: the solution used for injection in all instances was a 2% solution of hog's gastric mucin (National Biochemical Corporation, Cleveland, Ohio) in sterile saline.

The mice were used only for intraperitoneal investigation. One millilitre of the solution was injected into the peritoneal cavity of three mice and examinations made after two, four, and six days. A similar routine was followed after an initial injection of 2 ml. of liquid paraffin, which was designed to promote an active inflammatory exudate. Intraperitoneal injections of 2 ml. of the solution were also performed in one rabbit, at intervals of three days, for periods of two and three weeks.

Two millilitres of the solution were injected intradermally into the abdominal wall of one rabbit; the surrounding areas were examined microscopically at intervals of three and six days, and the site of injection was itself examined latterly.

Intravenous injections of 10 ml. of the solution were given in three rabbits daily over periods of two days, one week, and two weeks, and similar amounts were injected on alternate days over a period of three weeks.

From the animal subjected to intraperitoneal injection, the peritoneum and abdominal lymph nodes were examined microscopically. The skin and injection site were examined in the rabbit subjected to intradermal injection, and from those in which the solution had been given intravenously the heart and pericardium, the lungs and pleurae, liver, spleen, kidneys, peritoneum, and lymph nodes in both thorax and abdomen were examined histologically.

All the material was stained by haematoxylin and eosin, and the Hotchkiss modification of the periodic-acid-Schiff stain (McManus, 1946). The liver parenchyma was in addition stained by Best's carmine method.

Results

In spite of the large doses of material used, the animals' health remained good throughout the experiments, with the exception of early loss of appetite and malaise, which did not persist. The injected material stained adequately with the Schiff stain.

The material injected intraperitoneally in the mice excited little inflammatory reaction: after liquid paraffin, mononuclear cells and polymorphonuclear leucocytes were observed in moderate numbers, but there was no histological evidence that the mucin was being phagocytosed. In the rabbits, there was no loss of translucency or peritoneal exudate. The material was readily eliminated, and the abdominal glands were enlarged and showed small amounts of the material in the cortical sinuses.

The initial result of the intradermal injections was an acute inflammatory reaction in the neighbourhood of the material. Phagocytosis occurred readily, several large mononuclear cells in the neighbourhood showing a vacuolated cytoplasm, which was Schiff-positive. The injection site itself later became heavily infiltrated by chronic inflammatory cells.

The effects of intravenous administration were observed in all organs. After two injections on successive days, the most marked changes were

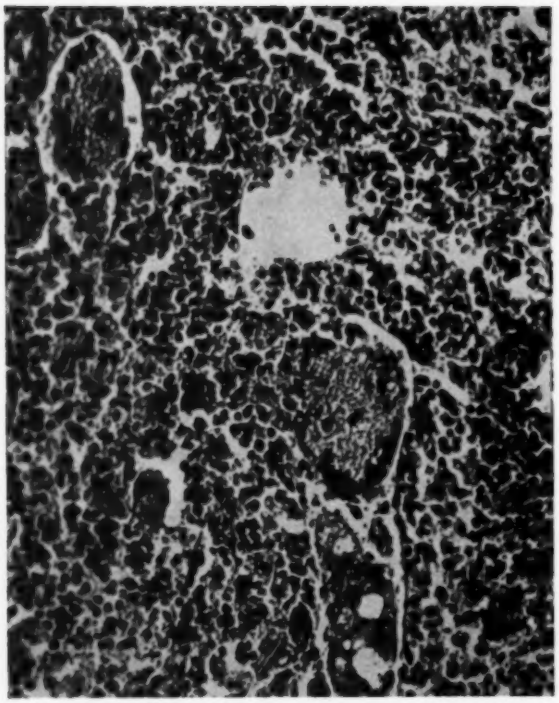

FIG. 6.—The rabbit spleen (×200) after injection of large amounts of intravenous mucopolysaccharide shows reticulum-cell hyperplasia forming giant-cells, and containing a foamy cytoplasm which is moderately Schiff-positive.

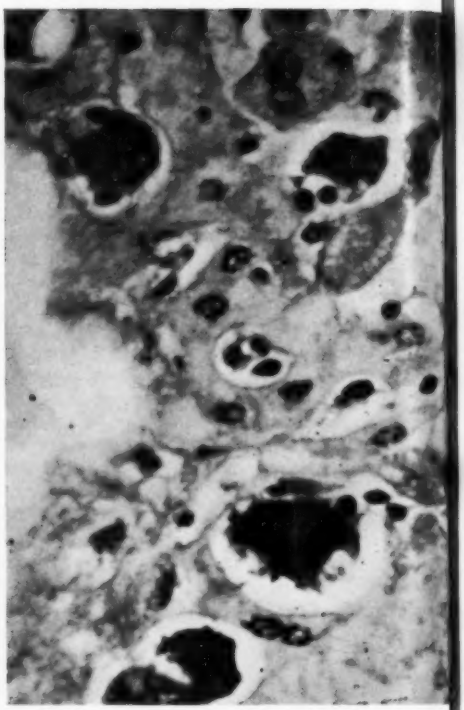

FIG. 7.—The rabbit liver (×500) shows similar hyperplasia of the Kupffer cells, which are incorporating large amounts of deeply staining Schiff-positive material. Smaller droplets, resistant to diastase, can be seen in the parenchymal cells.

observed in the kidneys which were hyperaemic, and showed swelling of the glomeruli and epithelium of the proximal convoluted tubules. In the liver there was moderate periportal acute inflammatory infiltration, and the Kupffer cells were swollen and vacuolated, containing droplets of Schiff-positive material (Fig. 7).

Observations on those animals injected for periods of one to three weeks differed only in degree. The lymph nodes in both thorax and abdomen were enlarged: moderate amounts of Schiff-positive material were present, apparently lying free in the sinuses. The liver showed droplets of Schiff-positive material in both the Kupffer cells and the parenchymal cells. In the kidneys there were small amounts of the material in the capsular spaces and in the tubular epithelium, but in the animals subjected to intermittent dosage there was little evidence of accumulation of the material.

The lungs showed thickening of the pulmonary interstitial tissue, which was infiltrated by mononuclears and granulocytes, and there was considerable hyperplasia of the pulmonary lymphoid tissue. The spleen showed marked hyperplasia of the Malpighian corpuscles in the animal subjected to the longest administration, and the pulp contained large numbers of multinucleated giant cells with a vacuolated cytoplasm (Fig. 6) which was moderately Schiff-positive. The appearances resembled those described by Hueper (1942a and 1942b) in connexion with the response to pectin.

In one of the final experiments, injections were stopped four days before examination and a marked diminution was observed in the amount of Schiff-positive material in the liver parenchyma and reticulo-endothelial system.

Examination of the serous membranes—pleura, pericardium, and peritoneum—failed to show evidence of inflammation, or aggregation of the mucus itself.

These results parallel very closely observations made previously by several authors on the tracing, storage, and disposal of polysaccharide material. The experiments are of interest in demonstrating the ability of the experimental animal to dispose of large quantities of mucopolysaccharide material, but in the context of the present study this attempt to explain the inflammation of the serous membranes was unsuccessful.

Another objective, however, to determine if it were possible to demonstrate the phagocytosis of circulating mucopolysaccharide, was successful. The giant cells seen in the spleen resemble the macrophages of Whipple's disease only superficially. But these appearances may have been modified by such factors as the duration of the experiment, dosage used, the organ involved, and the mode of administration. Further study along the same lines must clearly await the isolation and chemical identification of the material stored in this condition.

Discussion

After Black-Schaffer (1949) had demonstrated the character of the material in the macrophages, interest as to the pathogenesis of Whipple's disease was centred upon the steatorrhoea which is frequently a dominant feature of the condition as seen in hospital. Thus the idea of an "abnormal" or "abnormally split" fat in the intestine, with inflammatory obstruction of the mesenteric lymphatics, was postulated in various guises over a long period (Whipple, 1907; Gaertner, 1938; Korsch, 1938; Escudero, Mosto, and Landabure, 1945; Reveno, 1950). An abnormal "excretion" of fat directly into the intestine or mesenteric lymphatics had been suggested to explain the steatorrhoea by two groups of authors (Reinhart and Wilson, 1939; Sailer and McGann, 1942).

The many features of the fully developed picture can be explained on the basis of a wide-range "malabsorption" defect, but the advocates of the obstructive theory have found difficulty in explaining the observation of two authors (Whipple, 1907; Sailer and McGann, 1942), which was confirmed in Case 1 of the present series, that the thoracic duct has been patent at necropsy. The possibility does exist, however, that the site of obstruction lies in the mucosa itself: probably the most constant observation at necropsy is the finding of large quantities of Schiff-positive material in the jejunal villi, which are themselves grossly thickened by the presence, not only of this material, but also by large, circular fat vacuoles.

The same aggregations may be responsible for the constant finding of occult blood in the stools and hypochromic anaemia, by initiating constant slight haemorrhage into the lumen of the intestine, and also for the appearances in the mesenteric lymph nodes by causing a partial degree of obstruction at that site.

While most students of the condition are in agreement upon the general character of this material, its source has never been determined. A number of observers (Black-Schaffer, 1949; Puite and Tesluk, 1955) regard the fundamental abnormality as being in the mucus secretion in the upper jejunum. It is noteworthy, however, that in all material examined in the present investigation, combined alcian blue and periodic-acid-Schiff-staining shows what is apparently normal activity by the secreting epithelium, independent of the presence of the Schiff-positive granules which are quite sharply confined to the lamina propria of the mucosa and the underlying lacteals. This observation, together with the fact that incubation with hyaluronidase does appear to have some impression upon the material, suggests the possibility that the fundamental defect may lie in an alteration of the mucopolysaccharide ground-substance in this region. The idea of a genetic predisposition is strongly suggested by the constant age of onset, its great predominance in the male, and the fact that it has once been reported in brothers (Puite and Tesluk, 1955).

In conclusion, despite the absence of experimental confirmation in this work, the most likely explanation of the exacerbations of prodromal symptoms and the lesions in the endocardium and serous membranes is that they represent an inflammatory response to small amounts of the material in the circulation over a considerable period of time.

Summary

There is some evidence that the fundamental defect in Whipple's disease may be an alteration in the mucopolysaccharide ground-substance of the jejunal mucosa. Another case is recorded in which the characteristic macrophages have been found in widespread sites, and also incorporated in endocardial vegetations.

These findings, together with the character of the prodromal symptoms, may indicate an intermittent systemic dissemination of the Schiff-positive materials, and an attempt has been made to study the systemic effects of a circulating mucopolysaccharide in the experimental animal.

Whilst the finding of the characteristic Schiff-positive macrophages in the mesenteric lymph

nodes and jejunal mucosa is invariable and essential for diagnosis, the occurrence of these macrophages in peripheral lymph nodes, already noted, is confirmed. In this connexion, an indefinite "sarcoid-like" picture has been found sufficiently frequently to be suggestive in the correct context.

The severe steatorrhoea and "malabsorption" defects which often dominate the patients' final period in hospital are probably the result of excessive formation of the material causing obstruction at the mucosal site.

My thanks are due to several clinicians and pathologists who have allowed me to examine their material, or directed attention to possible sources, and in particular to Professor T. Crawford for advice and criticism and for undertaking all the photography included in the paper.

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CUTANEOUS PETECHIAE IN FATAL CORONARY ARTERY DISEASE

BY

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In the clinical recognition of myocardial ischaemia, the nature and distribution of the referred pain which frequently accompanies it are of considerable diagnostic importance. Unfortunately in cases of sudden death there may be no such history and not always is an unequivocal fatal cardiac lesion found, such as a recent or ruptured infarct, or an acute occluding lesion of a coronary artery. In some there is little to indicate why or how the patient actually died, as the only cardiac lesions found are of long standing. The fatal disturbance is often of function rather than of structure and in such cases the post-mortem diagnosis of acute coronary insufficiency is made by implication rather than observation.

In seven necropsies on cases of acute coronary death cutaneous petechiae were observed at sites commonly associated with referred pain of cardiac origin (Fig. 1). No such lesions were observed in any deaths not of coronary origin. These petechiae were all outside the area of cutaneous post-mortem hypostasis and in no case was there any evidence of generalized haemorrhagic disease. It is suggested that this sign may be of diagnostic significance in the post-mortem diagnosis of coronary insufficiency. There appears to be no previous description of similar observations in cases of coronary heart disease either before or after death.

Case Reports

Case 1.—A housewife aged 72 years died two days after admission to hospital in a collapsed state with dyspnoea and pain in the left shoulder. Three weeks previously she had experienced an attack of gripping pain in the retrosternal region. There was no previous history of any other illness.

At necropsy fine recent petechiae were seen over the left deltoid region and in the left submammary region. The heart showed extensive recent infarction involving the whole of the anterior wall of the left ventricle and most of the ventricular septum. The coronary arteries were severely atheromatous, and the first part of the anterior descending branch of the left coronary artery was occluded by a mass of degenerate atheroma.

Case 2.—A man of 64 years who had led a healthy and active life as a pedlar was found dead in bed the day after he experienced a sensation of being unwell, accompanied by pain in the chest and abdomen, although these did not restrict his usual activities.

There was a localized area of fine recent petechiae on the right side of the chest extending as a horizontal band 7½ cm. long and 5 cm. wide just below the right nipple and fading out over the right border of the sternum. The lungs showed acute oedema. The heart was not enlarged. A solitary subepicardial petechia was present in the atrioventricular groove near the right cardiac border. The right coronary artery showed a region of atheromatous stenosis at a point 1 cm. from the origin. Distal to this there was a segment of complete occlusion by a thrombus extending for 2 cm., and histological examination showed acute haemorrhagic extravasation into the adventitia overlying this occluded portion. The myocardium showed no evidence of acute infarction.

Case 3.—This 37-year-old blacksmith was found to have an enlarged heart and an atypical bundle-branch block at the age of 29. In the subsequent two years he had several attacks of "blackout" which were preceded by pain in the tips of the fingers of the left hand which spread up into the left arm, and terminated in unconsciousness. There was no history of convulsive fits. During the last two years of his life he suffered from palpitations.

Three days before death in April, 1953, he had increasing dyspnoea and epigastric pain radiating to the back between the shoulder blades, upwards into both sides of the neck, and down the inner sides of both arms. Clinical examination showed severe shock with cardiomegaly and venous congestion.

At necropsy numerous small recent petechiae were seen over both shoulders and between the shoulder blades. The heart was enormously enlarged, weighing 33 oz. (935 g.), this being due to massive hypertrophy and dilatation of the left ventricle. The atria were of normal size. Several superficial recent subepicardial petechiae were present in the atrioventricu-

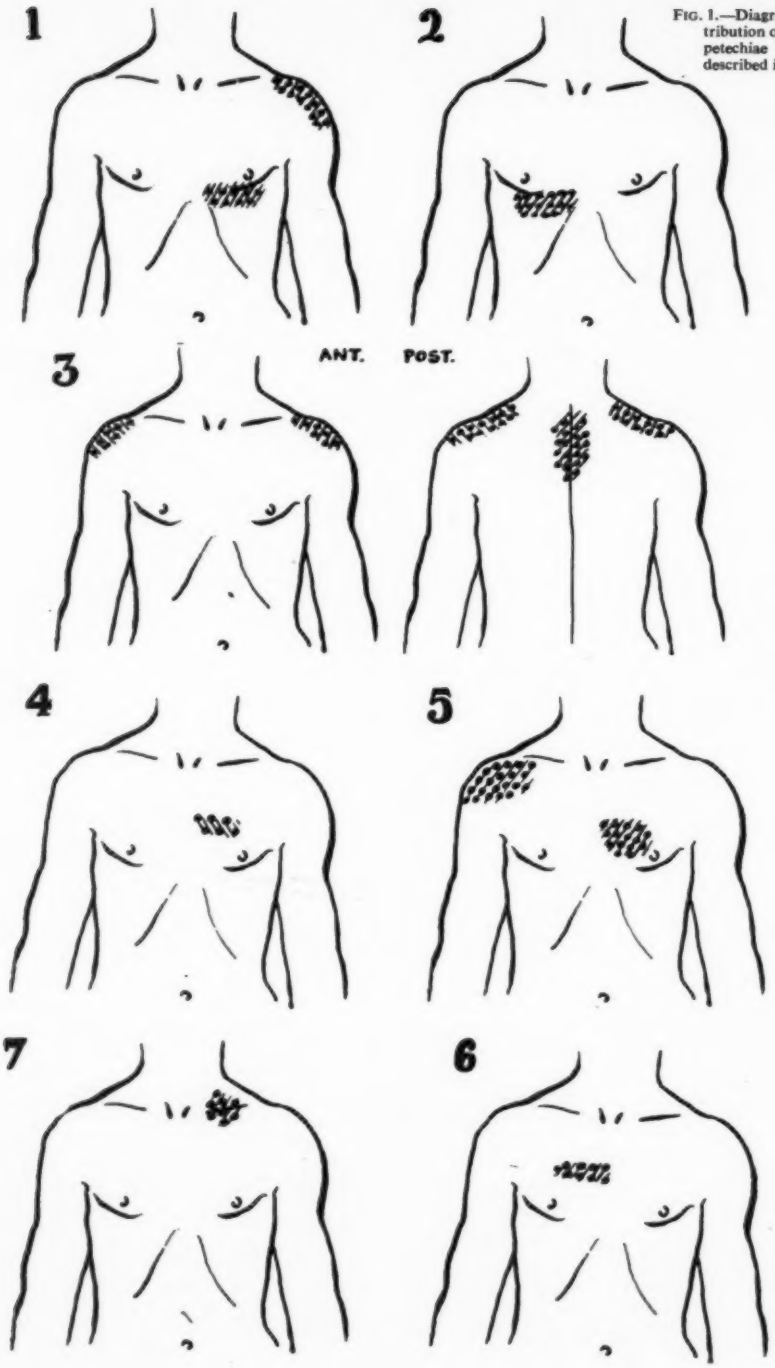

FIG. 1.—Diagram showing distribution of the cutaneous petechiae in the cases described in the text.

lar groove. There were no septal defects or valvular malformation and no stenosis at the aortic outlet. The aorta showed a moderate generalized hypoplasia. The coronary arteries shared in this hypoplasia ; the internal diameters of first parts of both main vessels measured only 2 mm., displaying a marked disproportion to the enormously increased mass of the myocardium.

Case 4.—A woman aged 87 years was found dead in bed having always enjoyed good health until two to three days previously, when she had complained of slight pain in the chest. This caused little distress, and she refused medical attention.

At necropsy six small recent petechiae were seen on the left side of the precordium. Internal examination showed terminal pulmonary oedema and moderate enlargement of the left ventricle. There was generalized coronary atheroma with a zone of recent occlusion in the first part of the right main artery. The myocardium was soft, but there was no actual infarct. Sections of the affected coronary artery showed old organized thrombus in the lumen and recent haemorrhage in the wall.

Case 5.—An 86-year-old man was found dead. There was no previous medical history. There were fine recent petechiae over the left side of the precordium, and over the anterior aspect of the right shoulder. Internal examination showed terminal pulmonary oedema and a moderately enlarged heart. The myocardium was soft and there were patches of interstitial fibrosis. The coronary arteries showed widespread stenosis by calcified atheroma, and there was recent occlusion of the right main artery by thrombus.

Case 6.—For several months this 68-year-old woman had suffered transient attacks of dyspnoea and a sensation of constriction in the chest which had caused very little inconvenience and responded to rest, aspirin, and hot-water bottles. Her final attack began in the usual way, but the sense of constriction became progressively worse and was accompanied by pain. She died before the doctor arrived.

At post-mortem examination there was a transverse, slightly oblique area of erythema over the upper portion of the right side of the precordium which stopped abruptly at the midline. Numerous petechiae were present in this area and there were a few up to 1.5 cm. beyond its well-defined margin (Fig. 2). Internal examination showed extensive calcified atheroma and stenosis of the main coronary arteries with complete occlusion of the anterior descending branch of the left artery from within 1 cm. of its origin. The myocardium was soft, but no infarct was found. Sections of the skin showed that the erythema was due to dilatation of the capillaries of the dermis, and that the petechiae were minute areas of recent haemorrhage.

Case 7.—A seaman aged 64 years was found dead in his bed. There was no available history of previous illnesses. There was a group of about 20

FIG. 2.—Post-mortem photograph of Case 6 showing the well-defined area of precordial erythema and petechiae.

petechiae occupying a circular area approximately 5 cm. in diameter in the skin overlying the central portion of the left clavicle.

Internal examination showed acute terminal congestion and slight oedema of both lungs, these changes being most severe at the bases. Both cardiac ventricles showed slight hypertrophy and there was a small number of subepicardial petechiae in the wall of the left atrium and along the inferior margin of the right ventricle. Both coronary arteries showed marked atherosclerosis with almost complete occlusion of the left coronary artery just proximal to its bifurcation, and of its anterior descending branch. There was an area of haemorrhagic extravasation into the adventitia of the circumflex branch beginning about 3 cm. from its origin and extending for ½ cm. This was confirmed by histological examination (Figs. 3 and 4). No massive myocardial infarction or fibrosis was present.

Discussion

Two of these patients (Cases 1 and 3) had been subjected to frequent clinical examinations before death, but in neither case had cutaneous petechiae been noticed, nor have similar lesions been recorded amongst the clinical manifestations of coronary disease.

Although fatal coronary disease is common, these petechiae are rare. Five of the seven cases were from a personally studied series of 360 deaths due to coronary insufficiency in which a search for petechiae was specifically made, giving an incidence of 1.7%.

It is considered that these cutaneous petechiae may be caused by intense efferent stimuli along a sympathetic nerve pathway similar to that involved in referred pain, possibly by agonal relaxation of blood vessels which had been

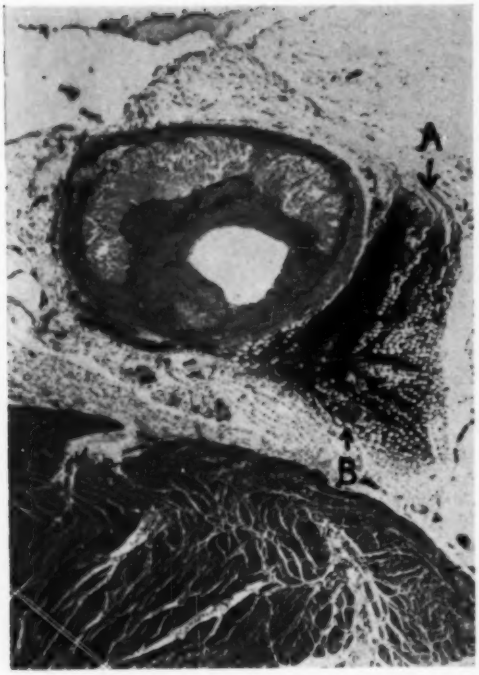

FIG. 3.—Section of the circumflex branch of the left coronary artery from Case 7 showing the adventitial haemorrhage. Involved nerves are indicated at A and B. (×12.)

FIG. 4.—The area indicated by arrow A in Fig. 3 ×150, showing two adventitial nerves in the area of haemorrhagic extravasation.

damaged by prolonged spasm. Peripheral vasoconstriction has been held to be responsible for referred cardiac pain by Roberts (1945) and has been demonstrated by variations of cutaneous temperature (Doret and Ferrero, 1951). An analogous mechanism is known to be responsible for the trophic lesions in the upper limb which are well-recognized clinical sequelae of acute cardiac pain and myocardial infarction (Askey, 1941; Kehl, 1943; Johnson, 1943; Steinbrocker, Spitzer, and Friedman, 1948; Hilker, 1949; Chitwood, 1950; Swan and Henderson, 1951).

Apart from possibly initiating reflexes, which may cause spasm of the peripheral vessels, the coronary arteries themselves are subject to vasospastic stimuli from various organs of the body (von Bergmann, 1932; Greene, 1935; Gilbert, Fenn, and LeRoy, 1940; Freedberg, Spiegl, and Riseman, 1944). It is possible that in the cases described in the present series the violent cutaneous reflexes were accompanied by equally violent spasm of the coronary arteries. This may explain why the petechiae have been found only at post-mortem examinations, and not in patients who have survived an acute coronary episode.

In four of the seven cases there were haemorrhages directly related to the coronary arteries themselves. In three of these there were haemorrhages in the coronary adventitia and direct involvement of the nerve elements may have played some part in the production of vasospastic reflexes to the skin and coronary arteries (Figs. 3 and 4). However, such haemorrhages are not common in fatal coronary insufficiency. In 100 cases without cutaneous petechiae, only three examples of adventitial haemorrhage were found and the adventitial lesions in the "petechiae" cases were probably an effect rather than a cause, occurring together with the cutaneous lesions by a similar reflex mechanism.

In Case 4 there was subintimal haemorrhage in the wall of the coronary artery, and, if it is considered that this condition may also be caused by nervous reflexes, this may have a bearing on a major mechanism of acute coronary insufficiency. Paterson (1936) first showed the importance of

subintimal haemorrhage in the pathogenesis of coronary disease, and Wartman (1938) considered it to be the second most common cause of coronary artery occlusion, thrombosis being the first. Expanding subintimal or mural haematomata are held to be responsible for about 10% of cases of acute coronary occlusion (Lowe, 1950), but in many more cases a small haemorrhage in the wall may play an important role by initiating thrombosis in the arterial lumen. Wartman (1950) found significant haemorrhage in 62% of a series of 365 cases of fatal coronary occlusion, and Drury (1954) found it in 37 of 58 cases (68%). In a personal series of 100 deaths due to coronary disease mural haemorrhage was found in 58 cases.

It is an interesting speculation that, if such haemorrhages can be produced by nervous stimuli, it is possible that anginal pain, not associated with organic coronary occlusion, might in fact cause such occlusion.

Summary

Seven cases are described in which cutaneous petechiae were found at necropsy on patients who had died of acute coronary insufficiency. These petechiae occurred in areas commonly associated with referred cardiac pain.

It is suggested that the presence of such localized petechiae is supporting evidence of acute coronary insufficiency, particularly in those cases where the coronary arteries themselves show no recent culpable organic lesion. In four of the cases haemorrhagic lesions were found in direct relationship to the coronary arteries. The mechanism is discussed and the possible role of reflex activity in the aetiology of subintimal coronary haemorrhages is considered.

I wish to thank Dr. J. D. H. Millar and Dr. M. Steel for permission to publish clinical details of their cases, and also Dr. J. M. Johnstone for the post-mortem findings on Case 7 and for his advice on the preparation of this paper.

Addendum

Since writing this paper the following additional case has been encountered.

A heavily built man aged 67 years was sitting quietly in his home watching television; he bent forward to place some wood on the fire, sat back in his chair and died suddenly and quietly.

At necropsy there was a small group of minute recent petechiae up to 0.5 cm. in diameter over the front of the left shoulder in an ill-defined horizontal band 5 cm. long and 1 cm. wide.

The heart was moderately enlarged (450 g.) and there was an extensive area of recent myocardial infarction involving the distal half of the anterior wall of the left ventricle and the adjacent portion of the interventricular septum.

The coronary arteries showed only moderate atheroma, except for an area of localized occlusion of the anterior descending branch of the left artery at a point of 3.5 cm. from its origin. Sections of this region showed considerable stenosis by a lipoid plaque in which there was recent haemorrhage which communicated with a recent thrombus that occluded the lumen of the vessel.

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CONGENITAL AGENESIS OF THE SPLEEN WITH CHRONIC ENTEROCOLITIS IN AN ADULT*

BY

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Persons with congenital absence of the spleen very rarely survive to adult life because they almost invariably have associated severe cardiovascular anomalies. There is no report in the literature of an adult who had chronic enterocolitis with secondary steatorrhoea in whom the possibility of absence of the spleen was suspected before death.

The exhaustive review of the subject of splenic agenesis by Putschar and Manion (1956) lists only 19 cases in adults. Fifteen of these had no other abnormalities, three had partial situs inversus, and one also had congenital disease of the heart. They also cite one case of a boy of 13 years, with no associated abnormalities, who died of injuries. Myerson and Koelle (1956) report a further case of an adult, with no other congenital abnormalities, who had a recurrent Waterhouse-Friderichsen syndrome. The case here reported is therefore the twenty-second case over the age of 3 years and the twenty-first in an adult.

An annotation in the *British Medical Journal* (1957) calls attention to this subject, there being an account in the same issue by Plowman (1957) of a case of a baby who, it should be noted, died from uncontrollable diarrhoea. The vast majority of the cases have been in babies, and, as France (1957) rather pertinently observes, "It would be surprising if it were not a relatively frequent incidental finding in any series of infantile deaths due to severe cardiac anomalies." Putschar and Manion (1956) list a total of 78 cases, a further 13 are quoted by Ivemark (1955), Bush and Ainger (1955) quote one, Towers and Middleton (1956) two, Nihoyannopoulos, Zannos, Oeconomon-Mavrou, and Statherou (1956) one, France (1957) three, Leadbeater (1957) one, and Fadell, Corbett, and Carrasco (1957) one. The case reported here is therefore the one hundred and third.

* Read at a meeting of the East Mercian Branch of the Association of Clinical Pathologists on March 30, 1957.

Case History

The patient was a man aged 72 years. In the 1914–18 war he had had an appendicectomy operation. In 1949 he had a *Salmonella tennessee* bowel infection. He was discharged from hospital with no diarrhoea, but shortly afterwards began to have diarrhoea with intervals of constipation which lasted three to four days. On admission to hospital on October 24, 1956, he had diarrhoea and his stools contained blood and mucus. He had no pain, but was losing weight rapidly. He was emaciated, had a dry, scaly skin, and his general condition was poor. On October 25 the blood urea level was 38 mg./100 ml. Haematological investigations on October 29 showed: Haemoglobin, 80% (11.8 g./100 ml.), white cells 7,000/c.mm. (total neutrophils 55% (3,850/c.mm.), band forms 15% (1,050/c.mm.), segmented forms 40% (2,800/c.mm.), eosinophils 1% (70/c.mm.), lymphocytes 37% (2,590/c.mm.), and monocytes 7% (490/c.mm.)). Examination of the blood film showed the red cells to be normocytic and some were a little hypochromic. A few of the red cells contained Howell Jolly bodies. (Subsequent examination of the film showed these to number about 1 per 1,000 red cells.) There was a moderate number of target cells. The presence of the Howell Jolly bodies prompted an inquiry to the ward as to whether he had ever had a splenectomy. When this was denied the matter was forgotten owing to extreme pressure of work. It can, therefore, be said with truth that the absence of this man's spleen was suspected.

Microscopy of the stool on October 24 and November 22 showed a moderate number of undigested muscle fibres and starch granules. No cysts, ova, or parasites were seen. On October 29 a histamine test meal showed no free acid. On November 9 a barium meal radiograph showed:

"Small bowel shows irregular spasm, locally dilated loops, and generalized deficiency pattern but no sign of obstruction. A 24-hour film showed that the small bowel has now emptied and there is a tendency to double contour throughout the whole colon with numerous apparent string signs and gross disorganization of the mucosal pattern of the distal colon, although the haustral pattern is relatively

preserved. This suggests a diffuse colitis, but I cannot see any evidence of carcinoma."

On November 9 the patient was too ill to be sigmoidoscoped. On November 20 the serum electrolytes were:

Sodium	...	312 mg./100 ml. (136 mEq/l.)
Potassium	...	11.8 mg./100 ml. (3.01 mEq/l.)
NaCl	...	618 mg./100 ml. (106 mEq/l.)
Calcium	...	8.2 mg./100 ml.

An examination of the fat content of the dried faeces on November 21 showed the total fat to be only 5.8%.

On November 23 the patient complained of pain in the hands and on examination had carpopedal spasm due to tetany. This responded well to an intravenous injection of 20 ml. of 10% calcium gluconate. On November 26 his hands were again in tetany and again treatment rapidly relieved it. On this day he was started on potassium chloride, 2 g. t.d.s. by mouth; he was found to have developed pneumonia and was started on a course of penicillin. On November 27 the haemoglobin was 69%—10.2 g./100 ml., and the serum contained:

Alkaline phosphatase		13.6 units/100 ml.
Potassium	... ...	13.9 mg./100 ml. (3.57 mEq/l.)
Calcium	... ...	6.7 mg./100 ml.

On November 28 examination of the dried faeces showed evidence of steatorrhoea. The findings were:

Neutral fat	3.1%
Free fatty acids	28.6%
Soaps	28.5%
Total	58.2%

On December 10 the serum potassium level had risen to 17.1 mg./100 ml. (4.4 mEq/l.) and on December 14 it was 28 mg./100 ml. (7.2 mEq/l.) and the serum calcium was 6.7 mg./100 ml. He died later that day.

Post-mortem Findings.—The body was that of a wasted old man. There was a paramedian scar in the right iliac region, but no scar was detected on the left side of the abdomen. There was acute tracheitis and bronchopneumonia with an abscess in the upper lobe of the right lung. The wall of the abscess was adherent to the right second costal cartilage. The heart was normal. The liver showed early cirrhosis. The spleen and splenic vessels were completely absent. This was confirmed by two observers. The gastro-intestinal tract showed only chronic enterocolitis; no other lesion was detected. Histological examination of the ileum and colon did not throw any light on the cause of the enterocolitis. The appendix was missing. Bacteriological examinations of a swab from the colon showed no organisms of the salmonella or shigella groups, and a swab from the lung abscess grew penicillin-resistant *Staphylococcus pyogenes.*

Discussion

The interest of this case lies not only in the record of a very rare congenital anomaly but also it possibly supplies evidence that chronic enterocolitis following a salmonella bowel infection may be due to lack of antibody formation by the spleen. It also raises the question of the diagnostic significance of Howell Jolly bodies in the peripheral blood.

It is not the purpose of this paper to review the vast amount of research that has been done to determine the role of the spleen in antibody formation. A few pertinent facts, however, must be considered. Perla and Marmorston (1935) review all the early animal experiments in a masterly fashion. Their conclusions are worth repeating:

"It is of interest that the resistance to histamine of the rat and mouse free from latent infections is unaffected by splenectomy. This suggests that the drop in natural resistance following splenectomy is not of a general character. It seems to be associated with those diseases and toxaemias which, in normal animals, affect the macrophage tissue of the spleen. The role of the spleen in resistance is due not entirely to the obvious capacity of its macrophage tissue, for phagocytosis and resistance may depend on many subtle chemical relationships which, at present, are little understood."

There is general agreement that, although the spleen has something to do with immunity, splenectomized human beings and also those with congenital agenesis or with atrophy of the spleen are not unduly susceptible to the common infections. It would appear that the reason for this is that in order to produce clinical evidence of an absence of the antibody-forming capacity of the spleen, the nature, dose, and route of entry of the antigen must be just right. Rowley (1950a) showed that in the splenectomized rat the production of circulating antibody is the result of the dosage of antigen and the route of administration. If the dose was small and intravenous there was a low antibody titre, but if it was intraperitoneal or intraportal the titre was as high as in similarly treated normal rats. Working with splenectomized human beings he showed (1950b) that when an intravenous antigen (heterologous erythrocytes) was given they failed to have a significant rise in antibody compared with normal controls. Motohashi (1922) showed that splenectomized animals had a poor antibody response when a small dose of intravenous antigen was given but a good response when the dose was large. He thought that only when the dose was large were other sites of antibody formation stimulated.

It is tempting to ascribe the patient's chronic enterocolitis to deficient antibody formation to a

small number of salmonella remaining in the intestine and its lymph nodes. This does not, at first sight, fit in with Rowley's (1950a) findings, but a salmonella infection is a generalized septicaemia and a small number of bacilli were probably in the systemic as well as in the portal circulation. The baby quoted by Plowman (1957) had uncontrollable diarrhoea, and one of the cases of atrophy of the spleen quoted by Boveri (1942) had fatty diarrhoea. No post-mortem examination was done on this latter case, and it could have been agenesis and not atrophy of the spleen. Engel (1939) stated that all published cases of uncomplicated non-tropical sprue showed atrophy of the spleen except for one, with the implication that the atrophy was the result of the intestinal disorder. It is also possible to postulate, however, that atrophy or agenesis of the spleen may be a factor in the causation of fatty diarrhoea.

It cannot truthfully be stated that the present case was diagnosed before death and no search for Heinz bodies was therefore made. After the patient died, however, the blood film made on October 29, 1956, was re-examined, the Howell Jolly bodies counted, and the film examined for Heinz bodies; none could, however, be detected. Absence of the spleen was, however, suspected because of the Howell Jolly bodies in the peripheral blood film. The target cells were not considered of diagnostic importance. Whitby and Britton (1957) give an account of the varieties of target cells together with the different conditions in which they are found. It should be emphasized that, according to Boveri (1942), the diagnostic significance of Howell Jolly bodies is limited. He quoted seven cases, one with beriberi, one with carcinomatosis of the abdomen and bone marrow, four with pernicious anaemia (one with associated cholecystitis), and one with chronic enterocolitis, all of which had normal spleens and yet showed Howell Jolly bodies in the red cells. It is possible, therefore, but unlikely, that the enterocolitis and not the agenesis of the spleen was the cause of the presence of Howell Jolly bodies in the red cells of the man described in this paper.

Summary

A patient is described with congenital agenesis of the spleen and splenic vessels who had chronic enterocolitis with secondary steatorrhoea. This enterocolitis followed a *Salmonella tennessee* infection seven years previously. He died of bronchopneumonia and a lung abscess. The possible immunological relationship of the absence of the spleen to the chronic enterocolitis and also the diagnostic significance of Howell Jolly bodies are discussed. This case is the twenty-second in patients over the age of 3 years and the twenty-first in an adult.

I wish to thank Dr. Stirling Eddie for permission to publish the case history and for his encouragement.

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METHODS FOR THE INVESTIGATION OF THE COAGULATION MECHANISM IN SMALL CHILDREN

BY

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The major problem which presents itself in any investigation of blood clotting in small children and infants is that of obtaining adequate specimens of plasma and serum. In the older child, as in the adult, blood may be taken by venepuncture in large amounts; the operation is technically easy and can readily be repeated if necessary. In the small infant the only veins readily available are those in the groin and the neck and the sagittal venous sinus. Venepuncture in these sites is much more difficult technically; it is not without hazard to the child and cannot readily be repeated. Therefore it has been necessary to develop methods for obtaining plasma and serum by skin puncture.

The first test of blood coagulation which was devised for use in clinical practice employed capillary blood (Wright, 1893), a matter of some interest in view of the poor opinion which is to-day held of techniques using capillary blood. Stefanini and Dameshek (1955), in their book on the haemorrhagic disorders, state that micro methods exist for the one-stage prothrombin time but that they are to be used only when blood cannot be obtained by venepuncture and the results obtained by these methods are to be regarded with grave suspicion. Objections to techniques involving the use of capillary blood are threefold. First, the use of capillary blood is opposed on the grounds that, in taking blood by this technique, the shed blood is to some extent mixed with tissue juices and the results are vitiated by the introduction of thromboplastic substances from this source. Merskey (1950) in a study of a series of haemophilic patients found that the consumption of prothrombin was reduced when blood for the test was taken by venepuncture, but that when blood for the test was taken by skin prick only 27 of 42 cases showed abnormal prothrombin consumption. Fantl and Sawers (1954), however, published a study of the blood clotting mechanism in a series of patients suffering from haemophilia and deficiency of plasma thromboplastin component (Christmas disease) in which they found that, while in patients suffering from classical haemophilia the consumption of prothrombin was reduced both in blood taken by venepuncture and by finger prick, in patients suffering from deficiency of plasma thromboplastin component the consumption of prothrombin was reduced in blood taken by venepuncture but not in blood taken by finger prick. These authors attributed this difference to the effect of admixture of tissue juices when blood was taken by finger prick, and suggested that tissue juices were capable of replacing plasma thromboplastin component but not antihaemophilic globulin. The method by which capillary blood was obtained from these patients was not stated, so that the extent of contamination of the specimens with tissue juice cannot be assessed, and it is difficult to understand how, if the tissue fluids, which are in a constant state of interchange with the plasma by the lymphatic circulation, contain a substance that can replace plasma thromboplastin component, the plasma can be deficient in this factor.

Technical objections to the use of capillary blood are that the amounts of plasma and serum available are small and that clotting takes place during the collection of the blood so that plasma free from serum cannot be obtained. These two objections can be considered together as they both depend on the method of obtaining capillary blood. If skin puncture is performed unskilfully and the finger or heel is subjected to compression in an effort to obtain an adequate flow of blood, the amount of blood obtained will be disappointingly small; it will be contaminated with tissue fluids and clotting at the end of the tube and on the skin surface will be troublesome. At the Alder Hey Children's Hospital, Liverpool, a wide variety of investigations, chemical, haematological, and serological, are carried out on small children and it is often necessary to repeat the tests at

frequent intervals for the control of treatment. Under these conditions methods using capillary blood have been developed for all investigations on babies and for many on older children; the collection of blood by skin puncture has been developed to a high degree of efficiency, and capillary blood is, in many instances in childhood, to be preferred to blood obtained by venepuncture from a small, struggling child.

The third objection to the use of capillary blood is that the methods of testing which are used are special micro methods and are basically unsound. Kato (1940) and Innes and Davidson (1941) described methods of determining the one-stage prothrombin time in which whole blood is mixed with an anticoagulant, either in a special slide or in a white cell pipette, and the test is carried out by mixing the whole blood with thromboplastin and calcium chloride in a watch glass or on a porcelain spot plate. Biggs and Macfarlane (1953) describe a method in which dilution of capillary blood is carried out in a white cell pipette. Approximately equal volumes of blood and anticoagulant are employed, the mixture is centrifuged, and the supernatant fluid used as diluted plasma. Both of these methods have disadvantages; in the former the test is a special "micro method" using whole blood, and it is difficult to read the end-point; in the latter the degree of dilution of the plasma depends to a considerable extent on the haematocrit, a higher concentration of plasma being present in the supernatant from anaemic patients.

The third type of method described is that of Bray and Kelley (1940): 1 ml. of blood is taken from the heel or finger and is allowed to fall into a tube containing anticoagulant. The plasma obtained by this means is adequate in amount and the dilution factor is the same as that when venous blood is used. Although Bray and Kelley described the method for use in the one-stage prothrombin time test, it has been used also in the thromboplastin generation test (van Creveld, Nagel, Nijenhuis, Miranda, and Tjon Sien Kie, 1954; van Creveld, Paulssen, Ens, and van der Meij, 1954), and its use has now been extended to the other tests of blood clotting.

The volume of blood which can be obtained by these methods is small by comparison with the volume obtainable by venepuncture. For routine laboratory tests micro methods may, however, safely be used (King, 1951) and the volume of blood plasma or serum is adequate for the tests without further modification: similarly, single tests of blood clotting in diagnosis and in the control of anticoagulant therapy can be carried out by the orthodox "macro" methods. When several tests have to be carried out on one specimen some further modification of the standard tests is necessary.

Methods

Collection of Specimens.—It is essential, when using blood taken by skin prick, to collect the specimens with great care. The main points in technique are: first, that the needle used must be sharp and the stab deep, in which case there is very little pain and only a minimum of restraint is required; secondly, the finger or heel must be warm, an important point in small babies in whom the peripheral circulation is very sensitive to a reduction in the external temperature; and thirdly, the blood should be allowed to fall directly into the test-tube or be collected into a capillary tube, accumulation of blood on the skin being avoided. Where these points of technique are followed a surprisingly large amount of blood can be obtained in a short time. If there is an adequate free flow of blood from a skin puncture, without compression of the part, the admixture of tissue fluids, after the first drop has been wiped away, is very small and may be disregarded for all practical purposes.

The instrument used is a clean Hagedorn needle which is sharpened frequently on a smooth stone, is wiped clean after use, and is kept in a bottle containing spirit. The needle is discarded when the edge cannot be resharpened or if any form of hook develops at the tip. The stab must be delivered sharply and cleanly at right angles to the skin surface and must penetrate deeply into the subcutaneous tissues. Any pushing or sawing action will cause trauma to the skin, will make the procedure needlessly painful, and will impair the quality of the specimen obtained.

In older children and in adults the subcutaneous pad of the tip of a finger or of the thumb is sufficiently large and vascular for capillary blood to be obtained in adequate amounts; in small infants the fingers are too small and the heel must be used. The skin of the heel remains soft and easily punctured until the child begins to stand and to walk about the cot and can readily be used up to about the age of 12 months.

Special preparation of a finger is usually unnecessary as constant hand movements in a warm environment maintain an adequate blood flow; in cold weather washing the hands in hot water will stimulate vasodilatation.

In the case of small infants all preparations for the collection of blood are made before the child is removed from its bed-clothes, the child is wrapped in a blanket with one foot exposed, and is held firmly by a nurse. This ensures that reflex peripheral vasoconstriction is kept to a minimum. Very small, immature, infants are warmed either in an incubator or with a hot-water bottle in the cot.

TABLE I
COMPARISON OF VENOUS AND CAPILLARY SPECIMENS

Test	Blood	Case							
		A	B	C	D	E	F	G	H
One-stage prothrombin time (whole plasma % prothrombic activity)	Venous	100	100	120	120	100	120	65	100
	Capillary	100	120	100	50	100	100	65	100
One-stage prothrombin time (diluted plasma % prothrombic activity)	Venous	112	107	63	107	120	100		
	Capillary	107	113	61	78	100	92		
Factor V assay (%)	Venous	80	100	75	110	130	100		
	Capillary	60	90	67	90	110	100		
„ VII „ „	Venous	85	120	120	120	140	120		
	Capillary	120	125	85	130	135	110		
Combined assay of prothrombin and factor VII (%)	Venous	110	105	105	110	110	110		
	Capillary	105	105	70	105	110	105		
Prothrombin assay (%)	Venous	80	90	65	100	100	100		
	Capillary	70	75	55	90	100	112		
Thromboplastin generation test (plasma %)	Venous	110	75	110	67	110	74	105	78
	Capillary	85	69	110	73	87	64	100	68
Thromboplastin generation test (serum %)	Venous	69	69	82	89	95	85	97	105
	Capillary	72	80	84	85	94	105	90	120

The skin is cleansed with spirit and allowed to dry and a sharp stab is made with only sufficient pressure on the part for the skin to be kept taut. The first few drops of blood are wiped away with sterile wool and discarded. Serum is obtained by holding the finger or heel so that the stab wound is dependent and allowing the drops of blood to fall directly into a plain glass test-tube or by allowing the blood to run into a glass tube of 2 mm. drawn out to a capillary point at one end. The blood is allowed to clot and serum is separated by centrifugation. Serum obtained by this technique is clear and free from haemolysis.

Clotting is prevented in blood from which plasma is to be obtained by the addition of 3.8% sodium citrate solution. A small tube (50×10 mm.) is marked at 1.0 ml., and 0.1 ml. of citrate solution is placed in it. Drops of blood are allowed to fall directly into the anticoagulant and are mixed by shaking the tube until the 1.0 ml. mark is reached. Blood is not allowed to fall on to the sides of the tube and the tube is not allowed to touch the skin. The specimen is discarded if any clotting takes place. Approximately 0.5 ml. of plasma can be separated from this specimen by centrifugation.

Tests of Coagulation.—Sufficient blood may be obtained by this method for the conventional one-stage assays of prothrombin activity (Quick, 1951), factor V (Stefanini, 1951; Wolf, 1953), factor VII (Owren and Aas, 1951), the combined effect of prothrombin and factor VII (Dreskin, 1952), and prothrombin alone (Owren and Aas, 1951). If several test must be carried out on one child the plasma can be diluted 1 in 5 with 0.85% sodium chloride solution and compared with diluted normal plasma.

The thromboplastin generation test (Biggs and Douglas, 1953) in its original form requires relatively large volumes of plasma and serum. It has therefore been modified in two respects. The incubation mixture of alumina plasma, serum, platelets, and calcium chloride need only be sampled once, at the end of six or 10 minutes (Biggs, Eveling, and Richards, 1955). This variation in technique permits the use of smaller volumes of each of the constituents of the reaction mixture, 0.1 ml. of each is adequate. Secondly, the method of preparation of alumina plasma is altered, the plasma may be diluted 1 in 5 with 0.85% sodium chloride solution before absorption of prothrombin and factor VII with alumina. These two modifications make it possible to carry out several assays of thromboplastin generation on a small blood sample.

A small series of experiments were undertaken to demonstrate the validity of these methods.

Specimens of plasma and serum were obtained from a series of volunteer donors by venepuncture and by skin prick. The one-stage prothrombin time was determined both on whole plasma and on plasma diluted 1 in 5 with saline. Assays of factor V, factor VII, prothrombin, and the combined activity of prothrombin and factor VII were carried out by the methods described above. The thromboplastin generation test was also carried out. The results of these experiments are set out in Table I.

Comparison of the results obtained in each of the tests using plasma or serum obtained by venepuncture and by finger prick show that there is little difference between the results and that the coagulation factors in the plasma and serum are normal by both methods. The results for the one-stage prothrombin time, though not for the other tests, in Case D show a significant difference between the values obtained on venous plasma and those obtained on capillary plasma. In this case the procedure was made difficult by the lack of co-operation from the donor, and by the thickness of the skin of the finger which made an adequate stab difficult. This illustrates the importance of good technique.

In addition two cases of haemophilia in small boys have been identified and confirmed by this technique.

A series of 17 specimens of plasma, three from normal individuals and 14 from newborn children, were used in the thromboplastin generation test. All were treated with alumina both before and after dilution. The results of this experiment, which are

TABLE II

COMPARISON OF RESULTS OF DILUTING PLASMA FOR THROMBOPLASTIN GENERATION TEST BEFORE AND AFTER ABSORPTION WITH ALUMINA

		Plasma Treated before Dilution (%)	Plasma Treated after Dilution (%)
Normal	1	84	95
	2	90	100
	3	100	108
Abnormal	1	74	82
	2	58	62
	3	80	130
	4	58	39
	5	95	106
	6	69	61
	7	73	90
	8	76	73
	9	73	65
	10	95	117
	11	82	81
	12	74	73
	13	79	61
	14	80	71

set out in Table II, show that good concordance between the two methods is obtained.

Summary

The difficulty of obtaining blood from small infants is discussed, and a method for obtaining citrated plasma by skin prick is described. The value of micro methods for tests of clotting function is discussed and it is concluded that the standard tests should be used rather than the micro methods.

Modifications to the thromboplastin generation test are described.

This work was undertaken in preparation for a thesis accepted by the University of Liverpool for the degree of M.D. I wish to express my thanks to Dr. E. G. Hall for his encouragement and advice, and to the laboratory staff of the Alder Hey Children's Hospital, Liverpool, for their help.

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THE EFFECT OF CITRATE ON EUGLOBULIN METHODS OF ESTIMATING FIBRINOLYTIC ACTIVITY

BY

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The time taken for the spontaneous lysis of the calcium-clotted fibrinogen of the isolated euglobulin fraction of plasma was used as a method of estimating fibrinolysis by Kowarzyk and Buluk (1950) and further developed by Niewiarowski (1952). This method has the advantage of requiring simple apparatus and taking a relatively short time to perform. It does not in any sense measure "natural" fibrinolysis, but it seemed worth while investigating and comparing it with other methods to see if it could be usefully employed in following variations in fibrinolytic activity in an individual and detecting differences between groups. In the course of this investigation the influence of the anticoagulant used became apparent.

Methods

Preparation of Plasma Specimens.—To eliminate as far as possible the effects of lipaemia (which inhibits fibrinolysis) without taking fasting specimens, the subjects (members of the laboratory and medical staff) took no fat with their breakfast. Blood was taken, between 9 and 10 a.m., by clean venepuncture with minimum stasis, using a silicone-coated wide-bore (20 S.W.G.) needle and all-glass syringe lubricated with silicone fluid MS 550. Samples of blood, each of 4.5 ml., were distributed quickly into 15 ml. conical centrifuge tubes (cooled in melting ice) containing (1) for oxalated plasma, 0.5 ml. of 0.1 M ammonium oxalate; (2) for citrated plasma, 0.5 ml. of 3.8% sodium citrate; (3) for "true" plasma, silicone-coated tube, no anticoagulant.

The tubes were spun at approximately 3,000 r.p.m. for 10 minutes, the centrifuge buckets for the "true" plasma tubes being previously cooled at 0° C. All blood and plasma specimens were kept in melting ice and tests were put up within 20 minutes of obtaining the specimen.

Euglobulin Lysis Time.—The method of Niewiarowski (1952) was used.

Reagents.—The following are required: 0.025 M calcium chloride, 1% acetic acid, borate solution (9 g. sodium chloride + 1 g. sodium borate made up to 1 litre with distilled water to give *p*H 9.0 as found by a glass electrode), and 0.1 M ammonium oxalate.

Procedure.—The determinations were carried out in 15 ml. conical centrifuge tubes. The tubes were soaked in chromic acid cleaning mixture for a few hours, brushed out, and soaked overnight in tap water, rinsed with distilled water, and dried in the oven before use. Scrupulous care in cleaning is necessary to achieve good duplication and tubes must all be of the same dimensions.

Using each of the three plasma preparations, duplicate tests were put up as follows:

To 9 ml. of distilled water in a centrifuge tube add 0.5 ml. of plasma. The *p*H is brought up to 5.3 by adding 0.1 ml. of 1% acetic acid to each tube. The tubes are then stood for 30 min. in the refrigerator at 4° C. for the euglobulin fraction of the plasma to precipitate. The tubes were then centrifuged for five minutes, the supernatant decanted, and the tubes drained by inversion on filter paper, when 0.5 ml. of the borate solution was added, and the tubes were placed in the 37° C. water-bath and stirred gently with a glass rod. Of the 0.025 M calcium chloride solution, 0.5 ml. was added to the resulting solution of euglobulins in borate and the time at which the mixture clotted was recorded. The tubes were left at 37° C. and inspected at intervals, and the lysis time determined. When the completion of lysis seemed near the clots were inspected every five minutes.

Fibrin Plate Method.—Fibrinogen free from citrate was prepared by the method of Laki (1951) from Armour bovine plasma fraction I. Plates were prepared from a 0.1% solution of fibrinogen by the adaptation of Astrup's method described by Holburn (1955). Unheated plates, for estimation of enzyme and activator, and plates heated for 30 minutes at 85° C. for estimation of enzyme only, were used. Plasma samples were prepared for estimation on plates by precipitating the euglobulin fraction at *p*H 5.3, as described above, and dissolving the precipitate in 0.5 ml. of the sodium diethyl barbiturate buffer, *p*H 7.8, used in the preparation of the plates.

Results

Euglobulin Lysis Time.—It will be seen from Table I that in all eight experiments the greatest activity was found in the citrated specimen. Oxalate and silicone plasma gave less active prepara-

TABLE I
COMPARATIVE EUGLOBULIN LYSIS OF PLASMA PREPARED IN VARIOUS WAYS

Sex	Age	Silicone Specimen		Oxalated Specimen		Citrated Specimen	
		Lysis Time	Rate	Lysis Time	Rate	Lysis Time	Rate
M	30	149	6·71	174	5·75	87	11·49
M	39	125	8·00	118	8·48	90	11·11
F	34	160	6·25	145	6·90	90	11·11
M	24	200	5·00	173	5·70	99	10·10
M	22	144	6·94	156	6·41	110	9·09
M	18	210	4·81	229	4·41	130	7·69
F	29	195	5·13	197	5·08	133	7·52
F	34	327	3·06	248	4·03	166	5·99
Average		189	5·74	180	5·85	113	9·26

Lysis time in minutes. Rate $= \frac{1{,}000}{\text{Lysis time}}$

tions, with no appreciable difference between them.

Citrate added in increasing amounts to a mixture of "true" plasma and distilled water before precipitating the euglobulin fraction by adding acetic acid to *p*H 5.3 gave the results shown in Fig. 1 where the increase in activity is proportional to the concentration of citrate in the mixture. Increasing quantities of oxalate also produced a slight activation but not to the same extent as citrate.

Heparin and "sequestrene" (di-sodium salt of ethylene diamine tetra acetic acid, E.D.T.A.) were also tried as anticoagulants. Neither was satisfactory; heparin plasma yielded a euglobulin solution that did not clot on the addition of calcium, and the euglobulin clots made from sequestrene plasma left a sludge in the bottom of the tube so that the completion of lysis was difficult to determine.

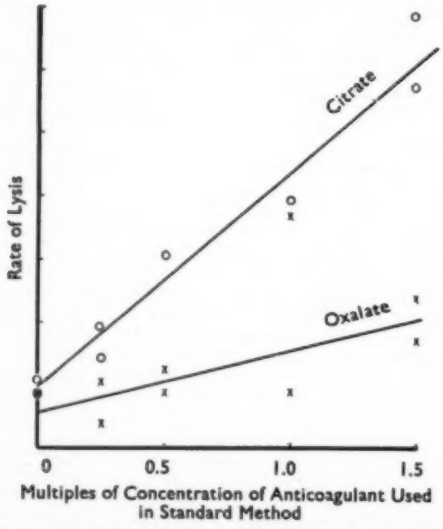

FIG. 1.—Effect of concentration of anticoagulant on euglobulin lysis rate.

Paper electrophoresis of euglobulin solutions gave no detectable albumin and showed the lipoprotein content to be reduced to about a third of that of the parent plasma.

Fibrin Plate Method.—Table II gives the comparative fibrinolytic activity by the fibrin plate method for five sets of plasma samples, and both the heated and unheated plates give the same order of activity as was found by the euglobulin lysis time method on a different series of specimens. No lysis was produced by 3.8% sodium citrate solution on heated or unheated plates.

TABLE II
COMPARATIVE FIBRINOLYTIC ACTIVITY BY FIBRIN PLATE METHOD (AREA LYSED IN MM.²)

Sex	Age	Silicone Specimen		Oxalated Specimen		Citrated Specimen	
		Heated Plate	Unheated Plate	Heated Plate	Unheated Plate	Heated Plate	Unheated Plate
F	34	6	46	6	30	20	125
F	34	No lysis	50	No lysis	45	39	79
M	24	Trace	20	14	25	35	68
M	24	16	49	12	52	45	64
M	24	No lysis	47	No lysis	42	60	145
Average		4·4	42·2	6·4	38·8	39·8	96·2

Discussion

Müllertz (1952) found that citrate added to the fibrinogen from which plates were prepared increased the activity of plasmin upon them. The results reported here show that citrate consistently increases the fibrinolytic activity in the isolated euglobulin fraction of human plasma as measured by two different methods.

The area lysed on a heated fibrin plate is a measure of the amount of proteolytic enzyme in the test solution. In the unheated plates plasminogen, destroyed by heating, is present and is converted to plasmin by activator in the specimen and the resulting lysis represents the sum of enzyme plus activator. The difference between the areas lysed on the unheated and heated plates gives the amount of activator and was about the same for oxalated and true plasma, 32.2 and 37.8 respectively, but was greater, 56.4, for the citrated samples. Thus there was an increase of activator as well as of pre-formed plasmin. This is not due

to citrate being carried over in the separation of the euglobulin fraction. No lysis was produced by six times as much citrate as was present in the plasma treated. Citrate may therefore be active in the transformation of proactivator to activator, which in turn activates plasminogen to plasmin, this increased plasmin content being shown on both the unheated and the heated plates and by the increased speed of lysis. Further work needs to be done to determine the precise site of action of citrate, but it appears that citrate should be avoided in the preparation of specimens for the estimation of fibrinolysis either by the euglobulin lysis time method or by the fibrin plate method, and that oxalate is suitable as it gives a result very close to that of true plasma.

The euglobulin methods deal with a simplified system in which the albumin anti-plasmin and much of the lipoprotein has been removed and do not represent the state of affairs in whole blood. It is doubtful how much of the activity in the euglobulin lysis time method is pre-formed plasmin and how much is formed from plasminogen during the test. Certainly when citrate is used this would appear to be possible. After several days' storage at 4° C. both oxalated and citrated plasma gave euglobulin clots that still lysed, although more slowly. No lysis was obtained by Bidwell's (1953) method on these stored samples.

The citrate effect found with euglobulin was not obtained when Bidwell's method was performed simultaneously with the work shown in Table I. Oxalated plasma was the most active in six out of eight tests, but the differences were not great enough for any conclusions regarding anticoagulant to be drawn. These variations of activity with anticoagulant make it imperative to stick to the same substance throughout any comparative series and to state what anticoagulant was used when recording results.

Summary

Citrate increases the fibrinolytic activity of the isolated euglobulin fraction of human plasma.

An increase in plasminogen activator as well as of active enzyme is produced.

Citrate should not be used in the preparation of specimens for fibrinolysis assay by the fibrin plate or the euglobulin lysis time methods.

Oxalate is a suitable anticoagulant for use in the above method.

I wish to thank Dr. F. A. Elliott, who initiated this work, for his help and encouragement, and the Research Subcommittee of Charing Cross Hospital for supporting the project; also Dr. A. L. Copley for calling our attention to the euglobulin lysis time method, Mrs. J. Pearson, B.Sc., for assistance in the earlier part of the work, and Dr. P. G. Bond for his help in the later part.

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THE KAOLIN CLOTTING TIME

A RAPID ONE-STAGE METHOD FOR DIAGNOSIS OF COAGULATION DEFECTS

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A major variable in blood coagulation tests is the nature and the amount of surface with which the blood comes in contact. When tests on re-calcified plasma are used the methods of preparation and storage of the plasma have also to be considered. The prolonged contact of plasma with a large surface, e.g., with glass powder or asbestos, is accompanied by the loss of many coagulation factors, including prothrombin and fibrinogen (Tocantins, 1945). Even a more limited exposure to glass, such as is likely to be encountered in normal practice, may be followed by considerable prolongation of the plasma clotting time (Margolis, 1957). The loss of clotting ability under these conditions is primarily due to the disappearance of the plasma thromboplastin antecedent (Rosenthal, 1955) which is the component most easily destroyed by contact with glass (Margolis, 1958).

An additional difficulty when dealing with plasma is that, as the number of platelets decreases with increasing centrifugation, the clotting time becomes more and more sensitive to variations in the platelet count and the results become more unpredictable.

Previous investigations (Margolis, 1957) indicated that the clotting time of re-calcified plasma can yield both sensitive and reproducible results when both the contact surface and the platelet content are controlled. The purpose of the present paper is to demonstrate the usefulness of this procedure for the diagnosis of plasma clotting defects.

Methods

The patient's plasma is re-calcified after the addition of different preparations derived from normal blood. Instead of relying on the walls of glass tubes as the activating surface, this is supplied much more effectively by the addition of a suspension of kaolin. By lowering the platelet content the system is made more sensitive, and the differences between normal and abnormal are magnified. The amount of platelet material is controlled by adding to rapidly-spun plasma a small standard amount of lysed platelets sufficient to mask the uncontrollable platelet activity left behind after centrifugation of the plasma samples.

All samples of blood and its derivatives are collected and handled in siliconed glassware unless otherwise stated.

Reagents.—The following are required :

(1) *Citrated Plasma.*—Venous blood is mixed with 20% trisodium citrate (0.2 ml. to 10 ml. of blood) and centrifuged at about 1,000 r.p.m. for 10 min. to obtain platelet-rich plasma. Platelet-poor plasma is obtained by further centrifuging at least at 3,000 r.p.m. for 30 min. and carefully removing the upper two-thirds of the plasma. The platelet count should not exceed 15,000/c.mm. It is important to prevent the formation of dried blood films on the walls of the centrifuge tubes, as this leads to the release of disrupted cellular material which is not easily sedimentable. A convenient method is carefully to transfer the citrated blood into a clean centrifuge tube, minimizing contact of blood with the tube above the level of the meniscus.

(2) *Aged Serum.*—Normal blood is allowed to clot in a glass centrifuge tube, incubated for several hours at 37° C., and then left for at least 18 hours at room temperature. The serum is decanted and centrifuged at 3,000 r.p.m. or more rapidly ; it is then transferred into siliconed tubes and may be stored at −20° C.

(3) *Al(OH)$_3$-Adsorbed Plasma ("Alumina Plasma").*—This reagent is prepared according to the methods of Biggs and Macfarlane (1957).

(4) *Heated Plasma.*—Normal plasma is heated at 56 to 57° C. for 20 min., and centrifuged to remove the denatured fibrinogen precipitate. A similar preparation heated at 60° C. is also useful.

(5) *Kaolin Suspension.*—Light kaolin, 200 mg., is suspended in 5 ml. of physiological saline.

(6) *Lysed Platelets.*—Platelet-rich plasma is centrifuged at 3,000 r.p.m. or more rapidly. The sediment is washed twice with saline and suspended in a volume of distilled water equal to that of the original plasma. The lysis is completed by freezing and thawing after which any remaining clumps are allowed to settle for a few minutes, and the super-

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natant is used. The platelet extract is then diluted with approximately 20 vol. of saline, preferably buffered with glyoxaline at *p*H 7.3 (Biggs and Macfarlane, 1957). The diluted extract is mixed with an equal volume of M/20 $CaCl_2$ immediately before use. This mixture provides platelet material equivalent to 5,000–20,000 platelets/c.mm., and the final concentration of the extract should be adjusted to give a clotting time of approximately 90 sec. with normal plasma. (After the first centrifuging a layer of platelets adheres to the sides of the tube and should be scraped off under saline with a wooden applicator. The platelet deposit is dispersed by vigorously rubbing in a drop of saline after the second and third centrifugations.) If it is desired to increase further the sensitivity of the system a higher dilution of the platelet extract is used, but this requires additional precautions (see below).

(7) *Alternative to Platelet Extract.*—As an alternative to the platelet extract, chloroform brain extract (Bell and Alton, 1954) may be used. The dilution must be empirically adjusted to give a normal clotting time of about 90 sec. (usually 1/1,000–1/10,000 of the stock suspension). This eliminates the somewhat time-consuming preparation of lysed platelets.

Procedure.—This is in two steps.

(*a*) *Screening Test.*—Into two glass clotting tubes ($\frac{1}{2}$ in. × $2\frac{1}{2}$ in. is a convenient size) are added in the following order:

Saline	0.05 ml.
Test plasma (patient's or normal)	0.1 ml.
Kaolin suspension	0.05 ml.

The tubes are incubated with occasional shaking for 2 min. at 37° C. and are then re-calcified with 0.1 ml. amounts of $CaCl_2$-platelet reagent. The samples are shaken at intervals and inspected for clotting. The end-point is sharp and is preceded by the kaolin flocculating coarsely 2–5 sec. before the formation of solid clot. In addition it is advisable to test the system for "platelet tolerance" by including two further tubes in which a mixture of equal parts of the patient's and normal plasma are re-calcified as above. In one of these tubes the platelet extract is replaced by saline. The clotting time without the platelets must be at least 30 sec. longer than the one with the extract (Table III). A difference of less than 30 sec. indicates the need for centrifuging the plasma further. This is particularly important when high dilutions of platelet extract are used as shown in Table II (B).

(*b*) *Full Test.*—The reagents (1–4) to be added to the test plasma are first diluted with equal volumes of saline. A series of clotting tubes is set up and the procedure carried out as in (*a*), saline being replaced with one of these preparations (Tables I and IV). With the aid of several stop-watches all the tubes can be dealt with at the same time. Alternatively the series may be split into groups of two or three tubes, provided these are tested within reasonable time of each other.

Since kaolin provides an internal activating surface, the clotting time is quite independent of the size of the containers and the absolute volumes of the reagents. These can be proportionally increased, if desired, to facilitate a more accurate measurement of the components.

Results

Reproducibility.—Table I shows the results of repeated tests on plasma from a patient under phenindione treatment. The figures show a high degree of reproducibility of the tests carried out within short intervals of each other and that there is little change in the clotting times after keeping all the reagents for one hour at room temperature. Leaving small volumes of diluted blood derivatives on the bench for periods longer than two hours should be avoided as they tend to lose their corrective activity.

TABLE I

KAOLIN CLOTTING TIME (SEC.) OF DINDEVAN PLASMA*

	Preparations Added to Dindevan Plasma			
Dindevan Plasma	Normal Plasma	Alumina Plasma	Aged Serum	Heated Plasma (56° C.)
130 (92)†	101	153	102	159
128 (93)	102	148	99	155
129 (95)	98	150	97	153
128 (91)	100	149	101	155

Samples of the patient's plasma, mixed with various preparations (25% v/v test plasma) and kaolin suspension, were re-calcified with $CaCl_2$-platelet reagent (see methods). The test was repeated four times within one hour to demonstrate the reproducibility of the results.

* "Prothrombin index" of 44.

† The figures in brackets refer to normal plasma tested in parallel.

Sensitivity.—Table II (A) shows the clotting times of mixtures of normal plasma and plasma from a severely affected haemophilic patient. Assuming the concentration of antihaemophilic globulin (A.H.G.) in the patient's plasma to be near zero, and that of normal to be near 100%, the mixture containing 25% A.H.G. can be confidently diagnosed as deficient. Clinically this

TABLE II

KAOLIN CLOTTING TIME (SEC.) OF MIXTURES OF NORMAL AND HAEMOPHILIC PLASMA

Patient	Concentration of Platelet Extract	Concentration of Normal Plasma in Mixture (%)				
		100	50	25	12½	0
A	1	93 95	102 100	136 132	152 155	570 550
B	1	95 92	95 98	110 113	125 122	205 198
	1:10	135 138	154 155	171 177	183 185	360 345

A = plasma from a high-grade haemophilic.
B = plasma from a moderately severe haemophilic.
In each test all five mixtures were re-calcified in parallel and a duplicate series was tested within 10 min. of the first one.

concentration of A.H.G. would represent a very mild bleeding tendency (Biggs and Macfarlane, 1957).

As was pointed out, the difference between normal and abnormal can be magnified by further reducing the number of platelets added to the system. The effect of this treatment is shown in Table II (B). The haemophilic plasma used in this experiment was obtained from a patient less severely affected than the patient in II (A). The test was performed with the usual strength and also with a 1 in 10 dilution of the same platelet extract. It is clear that the results are more informative when the diluted extract is used. To ensure that the plasma mixtures were sufficiently free of platelets the clotting times with and without added platelets were compared as described above. As shown in Table III, the clotting time of the saline control was well in excess of that with the added extract and the conditions of the test were therefore satisfied.

TABLE III
KAOLIN CLOTTING TIMES WITH VARYING CONCENTRATIONS OF PLATELET EXTRACT

Concentration of platelet extract (%)	100*	33	11	3·7	0
Clotting time (sec.) ..	98	123	145	169	203

The plasma used was the 50% mixture of normal and haemophilic plasma shown in Table II (B).
* Standard diluted extract.

Differential Diagnosis of Coagulation Defect.—The type of clotting defect is identified by assessing the corrective effect of added reagents prepared from normal blood. Examples of tests on patients belonging to different groups are shown in Table IV. Haemophilia is corrected by alumina plasma and Christmas disease by serum. Neither is corrected by heated plasma (56° C.), which fact distinguishes these conditions from the plasma thromboplastin antecedent (P.T.A.) deficiency and the Hageman trait (Ratnoff and Colopy, 1955). The distinction between the latter two is not quite as clear, as the Hageman trait is partly corrected but not the P.T.A. defect by plasma heated at 60° C., but this is hardly likely to present a practical difficulty as the Hageman trait is a very rare condition and it presents an entirely different clinical picture. (For further information see Biggs, Sharp, Margolis, Hardisty, Stewart, and Davidson, 1958; Margolis, 1958.)

TABLE IV
KAOLIN CLOTTING TIME IN SOME HAEMORRHAGIC CONDITIONS (SEC.)

Test Plasma	Preparations Added to Test Plasma					
	Patient's Plasma	Normal Plasma	Alumina Plasma	Aged Serum	Heated Plasma (56° C.)	Heated Plasma (60° C.)
Normal ..	—	90	85	70	92	—
Haemophilic (moderate)	360(90)*	120	132	238	240	—
Christmas disease (mild)	160(95)	122	169	100	168	—
P.T.A. deficiency (mild)	195(83)	114	118	76	116	200
Hageman trait	1,320(90)	99	97	90	105	350

* The figures in brackets refer to normal plasma tested in parallel.

The range of the added preparations can be modified to suit individual cases and extended by the inclusion of plasma from cases with known coagulation defects (direct matching).

Discussion

The theoretical considerations, with particular reference to the effect of contact, on which the methods are based, have been discussed elsewhere (Margolis, 1957). It has since been demonstrated that both the Hageman factor and P.T.A. are concerned in the activation of clotting by contact (Biggs *et al.*, 1958). It is therefore not surprising that in the absence of satisfactory control of contact conditions the results of clotting tests in cases of deficiency of these factors were often difficult to interpret. What may not, at first, appear obvious is why it should be necessary not only to control but also to reduce the platelet concentration in order to increase the sensitivity of the system to deficiency of A.H.G. and Christmas factor. One might expect that an arrangement where all the components except the one being assayed are kept within normal limits would be quite satisfactory, since the latter would become the limiting factor in the reaction. The clotting time is, however, a resultant of a complicated chain of successive overlapping reactions. The phase of coagulation in which A.H.G., Christmas factor, and platelets are directly involved probably represents only a fraction of the total clotting time. When the platelet concentration is greatly reduced and the clotting time thereby increased, it is presumably this phase of the reaction which is selectively lengthened. Under these conditions any deficiency of A.H.G. or Christmas factor which is reflected by a further prolongation of the relevant stage of the reaction will have a proportionally greater effect on the total clotting time. The present results are in agreement with the observations of Quick (1951) that the difference between the clotting time of normal and haemophilic plasma is magnified by using rapidly centrifuged specimens of blood. By extending this argument, a similar increase in the sensitivity of

the method could be expected if, instead of platelets, other components of blood thromboplastin were reduced. This is, in fact, borne out by a device often used in the diagnosis of mild coagulation defects, where the plasma of the suspected patient is diluted in the plasma of a severely affected case. This method has been used for the assay of A.H.G. by one-stage clotting procedures (Soulier and Larrieu, 1953; Langdell, Wagner, and Brinkhous, 1953), but suffers from the disadvantage of requiring a stock of suitable abnormal plasma as one of the reagents. Moreover, the results of these methods showed considerable variations between different experiments, presumably owing to the inadequate control of the contact surface.

The present method appears to be free from these drawbacks, the reagents being readily available and the results reproducible from day to day. The sensitivity of the system can be adjusted at will and is apparently limited only by how effectively platelets are removed from the plasma. In addition, the technique is simple and, being based on clearly defined principles, can be easily modified to suit individual requirements.

Summary

A simple, one-stage method for the detection and diagnosis of plasma coagulation defects is described. The test is based on the clotting time of re-calcified plasma in which the conditions are controlled by the addition of kaolin and a diluted platelet or platelet-substitute reagent. The type of deficiency is determined by assessing the corrective effect on the clotting time of the abnormal plasma of various derivatives of normal plasma. The procedure appears to be adequate for detecting even mild degrees of deficiency of plasma clotting factors.

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THE DISTRIBUTION OF CELLS IN PERIPHERAL BLOOD SMEARS

BY

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The factors influencing the distribution of cells in smears are complex. The specific gravity and the size of the cells, the surface tension, capillarity and viscosity of the fluid medium, and the driving force of smearing all play a part in distributing the cells (Schill, 1936; van den Berghe and Blitstein, 1946). Stickiness of the cells has been quoted as a possible factor (Dacie, 1956).

In a given preparation the fluid medium and the driving force are factors which are common to all the cells and the only two important known variables are the specific gravity and the size of the cells.

The object of the present study was to determine whether one of the variables played a dominant role in determining the distribution of the cells in peripheral blood smears. It is generally observed that the larger cells tend to collect at the very edge and tail of the smear, but no general principle has been formulated.

Method

Peripheral blood smears were made from four patients suffering from chronic myeloid leukaemia. Each smear was divided into blocks 2–3 mm. square, the dividing lines running parallel to the edges of the slide. Differential counts were carried out as follows: (1) Five hundred consecutive cells were examined in each block or area. The distribution of 20,000 cells in four slides was studied. (2) The blocks were selected so that four were placed in the proximal half of the smear and four in the distal half of the smear. In each half two blocks were placed in the central part of the smear and two blocks at the edge of the smear. (3) Counts were done at the tail of the smear and at the very edge, the latter being defined as a track along the edge of the smear no greater than the diameter of an oil immersion field.

Method of Analysis of the Differential Counts

The results were analysed in two planes—namely, the centre/edge and the proximal/distal plane.

The absolute cell counts for each cell type in all the centre blocks of all the smears were totalled. The edge, proximal, and distal blocks were treated in the same manner. In the case of the very edge and tail counts the results were multiplied by 4 to standardize the total counts for each region to 8,000 cells.

Eosinophils are not included in the analysis as the detailed differential counts were numerically inadequate.

Results

The differential counts are set out in Table I with the ratios in Table II and Fig. 1.

With the scheme adopted, the ratios above 1 indicate the tendency of the cell to occur maxi-

TABLE I

DIFFERENTIAL COUNTS

All Patients Absolute Figures	Lympho-cytes	Basophils	Blast Cells	Neutrophil				Eosinophils
				Pro-myelocytes	Myelocytes	Meta-myelocytes	Segmented	
Centre blocks..	255	219	1,207	866	856	3,394	891	154
Edge blocks ..	182	194	1,295	980	903	3,246	882	152
Very edge ..	12	19	343	376	240	748	189	184
Proximal blocks	263	222	1,269	859	868	3,335	922	139
Distal blocks ..	174	166	1,233	1,037	941	3,305	851	177
Tail	12	16	361	413	239	703	162	25

TABLE II

RATIOS

Ratio	Lympho-cytes	Basophils	Segmented Neutro-phils	Meta-myelocytes	Myelocytes	Blast Cells	Pro-myelocytes
Centre, edge	1·40	1·13	1·01	1·05	0·95	0·93	0·88
Centre, very edge ..	5·31	2·88	1·18	1·13	0·89	0·88	0·58
Proximal, distal ..	1·51	1·34	1·08	1·01	0·92	1·03	0·83
Proximal, tail ..	5·50	3·47	1·42	1·19	0·91	0·88	0·52
*Mean (μ) size of cell	6·0	10·2	10·2	10·2	12·9	13·3	16·3
†Specific gravity ..	1·063	1·070	1·080	1·080	1·070	< 1·070‡	< 1·070‡

* = Thorell (1947), Kristenson (1949).
† = Graham *et al.* (1955).
‡ = Davidson (1957).

mally at the central and proximal part of the smear. Conversely, the ratios below 1 indicate the tendency of the cell to occur maximally at the edge and distal part of the smear. The amount above or below unity is also an expression of the degree to which the tendency is present.

The following pattern is present (three discordant figures):

(1) In all cases the centre/edge and the proximal/distal tendency is accentuated in the centre/very edge and the proximal/tail ratios respectively.

(2) The lymphocytes, basophils, segmented neutrophils, and metamyelocytes tend to occur maximally at the central and proximal parts of the smear (ratios above 1) and the myelocytes, promyelocytes, and blast cells at the edge and distal parts of the smear (ratios below 1).

(3) The degree to which the tendency is present can be correlated directly with the mean diameter of the cell.

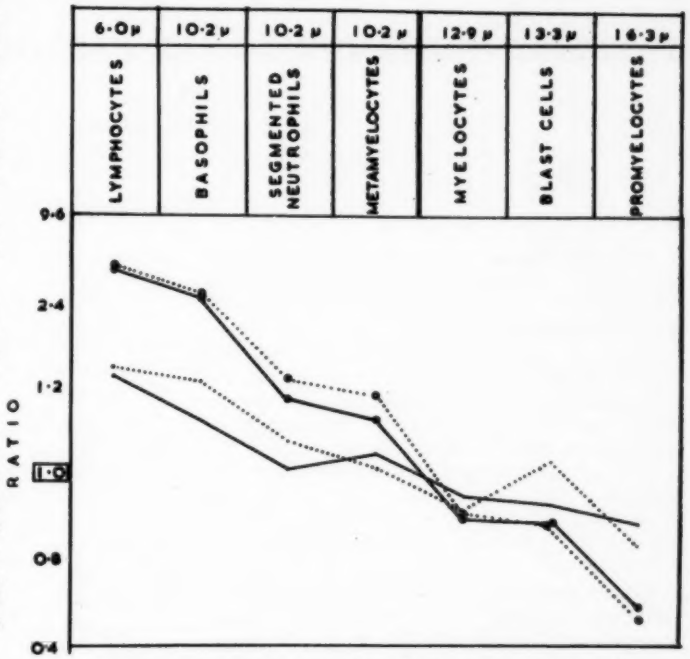

FIG. 1.—● . . . ● proximal/tail ratio. ●——● centre/very edge ratio. . . . . proximal/distal ratio. ——— centre/edge ratio.

Discussion

The arrangement of the cells according to size in this correlation is based on the generally accepted mean diameters of the white cells in peripheral blood smears (Wintrobe, 1956; Bessis, 1956; Darmady and Davenport, 1954; Whitby and Britton, 1953; Albritton, 1952; Undritz, 1952). In the process of smearing, the concern is with the size of the living cells. There is no evidence to suggest that the relative size of the living cells differs from that seen in peripheral blood smears (Thorell, 1947; Kristenson, 1949). There is also no evidence to suggest that the relative size of the cells is altered in chronic myeloid leukaemia.

Summary

The factors involved in the process of smearing are complex, but size alone is the dominant factor determining the distribution of cells in peripheral blood smears.

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FAMILIAL HAEMOPHILIA AND FACTOR VII DEFICIENCY

BY

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This investigation is reported because of the combination of familial haemophilia and factor VII deficiency and the unusual occurrence of a female haemophiliac.

Cases of combined deficiencies of different clotting factors are extremely rare and up till now there have been reported combinations of haemophilia and factor V deficiency (Koller, 1954), haemophilia and Christmas disease (Soulier and Larrieu, 1953), Christmas disease and factor VII deficiency (Bell and Alton, 1955; Stein and Abrahams, 1956; de Vries, Kettenborg, and van der Pol, 1955), but not of haemophilia and factor VII deficiency.

Female haemophiliacs in the homozygous state have been reported by Merskey (1951), Israels, Lempert, and Gilbertson (1951), and in the heterozygous state by Taylor and Biggs (1957) and Fantl and Margolis (1955), their fathers being completely normal.

The patient reported here is apparently in the heterozygous state since her father had no bleeding phenomena during his life.

Family History

Colin Br. (aged 11) bruised easily when an infant. He was admitted to hospital for the first time in 1949, when 3 years old, with a haematoma of the right calf. On admission in 1951 a diagnosis of haemophilia was made in view of the prolonged plasma clotting time and reduced prothrombin consumption. He was readmitted several times afterwards with haemarthroses of knees and hip.

He was first seen at this hospital in March, 1957, when he was admitted with bruises on his legs and severe prolonged bleeding from the gums. Routine investigations showed only an unexpectedly prolonged prothrombin time (20/14 sec.). He was transfused with 2 pints of fresh A rhesus-negative blood and given vitamin K_1. Next morning the prothrombin time was 15/13 sec. and bleeding was diminished. Several teeth were then extracted and treated in the following days and he was discharged well. Three months later he was readmitted with a few bruises on the legs and bleeding gums. Bleeding subsided with local treatment and vitamin K_1.

Brenda, his sister (aged 10), appears normal without any haemorrhagic manifestations up till now.

Eileen Br., his mother (aged 46), revealed that she had had excessive bleeding during both her deliveries, during and after a hysterectomy, being transfused in order to control haemorrhage, and after tooth extractions at different times.

Frank G. (aged 43), his maternal uncle, has bled since a small child after trauma and tooth extractions, oozing from sockets usually persisting for about three days. In 1948 he had a blow in the abdomen which was followed by severe haematemesis and melaena and he was transfused in order to control the haemorrhage.

Frank G.'s daughter was not available, but according to her father she has no bleeding phenomena.

Cathleen M. (aged 49), his maternal aunt, and her three children, two males and one female, of whom only one male (David) was examined, have no bleeding phenomena.

His grandmother and grandfather had no bleeding phenomena, were not relatives, and in his grandfather's family there were no bleeders. His grandmother's brother was a bleeder and bled to death after trauma. His grandmother's sister was said to be an actual bleeder, but the available evidence was conflicting. Her son died when a child from excessive bleeding.

The pedigree tree (Fig. 1), showing the inheritance of haemophilia and factor VII deficiencies, is based on investigations of the available members of the family. None of those without bleeding history was available for examination.

Laboratory Investigations.—The clinical history of the family is consistent with haemophilia, but the prolonged prothrombin time and the female bleeder made the diagnosis doubtful. The confirmation of the haemophilia and the investigation of the prolonged prothrombin time were therefore necessary. From Table I the bleeding time is obviously normal, while the clotting time (Lee-White mean of four tubes) is slightly prolonged in Eileen Br. and Frank G.

Since 1949 repeated estimations of Colin Br.'s clotting times ranged between 4 and 14 minutes, the pla-

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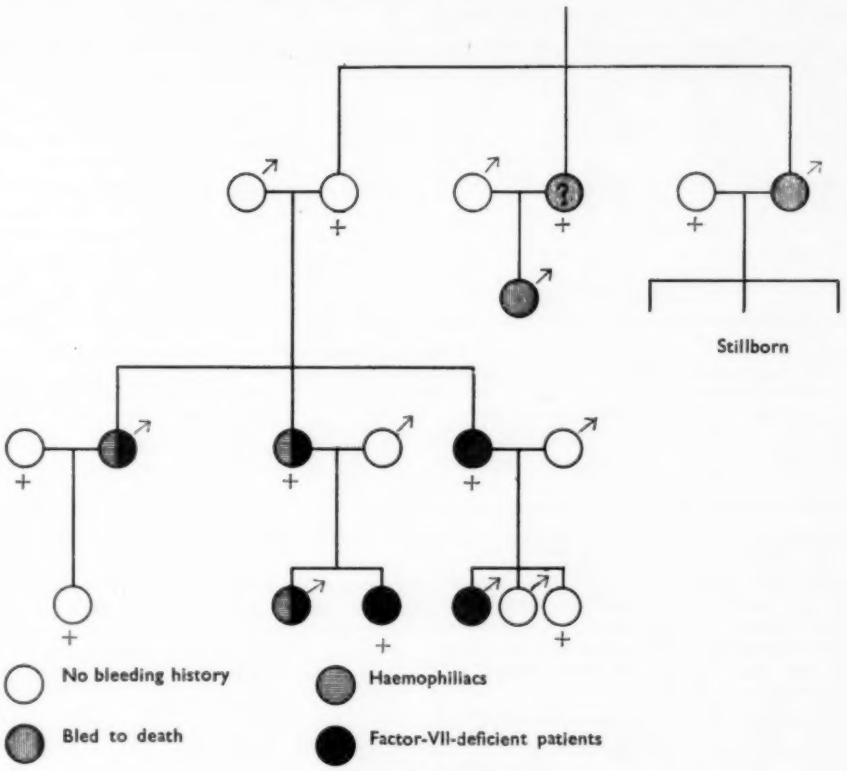

FIG. 1.—Pedigree tree.

lets between 250,000 and 350,000 and the fibrinogen between 0.18 and 0.31%. The prothrombin time is slightly but definitely prolonged, and Colin Br.'s ranged from 17 to 20 sec. (control 13–14 sec.) on different occasions. The prothrombin time is not corrected with the addition of fresh normal alumina-adsorbed plasma in 10% quantities to the patient's plasma. Alumina plasma contains adequate amounts of factor V but no prothrombin or factor VII which have been adsorbed by the alumina. A factor V deficiency should therefore be excluded. The prothrombin time is corrected by adding normal stored serum in 10% quantities to

TABLE I

RESULTS OF COAGULATION STUDIES OF THE FAMILY

	Clotting Time (min.)	Bleeding Time (min. sec.)	Prothrombin Time (sec.)	Prothrombin Time when 10% Normal Absorbed Plasma Added (sec.)	Prothrombin Time when 10% Normal Serum Added (sec.)	Factor VII (%)
Eileen Br.	15	1 30	16·5	15·5	12·5	60
Colin Br.	13·5	1 45	18*	18	14	40
Brenda Br.	9·5	3 30	19	18·5	13	40
Cathleen M.	11·5	2 15	16·5	16	12·5	—
David M.	10·5	1	18	17	13·5	45
Frank G.	17	1 15	17	17	13	50
Normal	6–13	1–4	13			80–120

* His prothrombin time was 20 sec. during an episode of bleeding.

TABLE II

MIXTURES OF PATIENTS' PLASMA AND SERUM WITH DINDEVAN PLASMA AND FACTOR-VII-DEFICIENT SERUM AND EFFECT ON PROTHROMBIN TIME

Eileen Br. Plasma (%)	Colin Br. Plasma (%)	Dindevan Plasma (%)	Eileen Br. Serum (%)	Colin Br. Serum (%)	Normal Serum (%)	Factor VII-deficient Serum (%)	Prothrombin Time (sec.)
100							16·5
	100						18
		100					45
90					10		12·5
90						10	17
	90				10		13·5
	90					10	18
		90	10				22
		90		10			24
		90			10		16·5
		90				10	37

TABLE III

THROMBOPLASTIN GENERATION IN NORMAL PLATELETS AND PATIENTS' SERUM AND PLASMA

Minutes	Normal Control (sec.)	Eileen Br. (sec.)	Colin Br. (sec.)	Brenda Br. (sec.)	Cathleen M. (sec.)	David M. (sec.)	Frank G. (sec.)
2	11	20	32	11	11·5	11	30·5
3	9·5	19	28	11	11	11	30·5
5	9·5	19	28	10·5	10·5	11·5	25

the patient's plasma. Normal serum contains factor VII but no factor V and only minimal amounts of prothrombin, both being consumed during clotting. Normal serum contains Christmas factor as well, but lack of this factor does not influence prothrombin time. The amount of prothrombin was normal as estimated with the two-stage technique (Biggs and Macfarlane, 1957) in two patients.

It is probable, therefore, that the prolonged prothrombin time is due to factor VII deficiency. This is established because (*a*) the low level of factor VII as determined quantitatively by Owren's method, (*b*) the failure to correct the prothrombin time of the patients' plasma when they were crossed with factor-VII-deficient serum, and (*c*) the only partial correction of "dindevan" plasma lacking in factor VII when crossed with the patients' sera (Table II).

Thromboplastin generation (Biggs and Macfarlane, 1957) was abnormal in three members of the family (Table III). In these three patients, by replacing their plasma with adsorbed normal plasma containing antihaemophilic globulin and factor V, or by adding minimal amounts of pure A.H.G. to their plasma, the generation of thromboplastin became normal (Tables IV and V). Mixtures of patients' plasma and known haemophilic plasma did not correct the deficient thromboplastin generation. No anticoagulant has been detected after the incubation of the plasma with normal plasmas or A.H.G. at 37° C.

These results of the thromboplastin generation exclude again factor V and Christmas factor deficiency which might have been responsible for the abnormal generation and establish the antihaemophilic globulin deficiency. They show in addition that factor VII does not influence at all the thromboplastin generation because the latter is normal when using normal plasma and patient's serum deficient in factor VII.

Treatment.—During the acute bleeding fresh blood was given and the haemorrhage controlled. Pure animal anti-haemophilic globulin should be reserved for severe bleeders and serious operations, having in mind the sensitivity that develops and the difficulty of its preparation (Macfarlane, Mallam, Witts, Bidwell, Biggs, Fraenkel, Honey, and Taylor, 1957). Vitamin K_1 was given as well, and in one other instance, when the bleeding was mild, only vitamin K_1. Although it is said to be without any benefit in congenital cases, Newcomb, Matter, Conroy, DeMarsh, and Finch (1956), van Creveld, Veder, and Blans (1956), and Stefanovic, Milosavljevic, and Stefanovic (1955) observed some beneficial response.

TABLE IV

THROMBOPLASTIN GENERATION IN NORMAL PLATELETS, PATIENTS' SERUM AND NORMAL PLASMA

Minutes	Eileen Br. (sec.)	Colin Br. (sec.)	Frank G. (sec.)
2	11	11	12
3	10	10	12
5	10	10	13

TABLE V

THROMBOPLASTIN GENERATION IN NORMAL PLATELETS, PATIENTS' SERUM AND PATIENTS' PLASMA + MINIMAL AMOUNTS A.H.G.

Minutes	Eileen Br. (sec.)	Colin Br. (sec.)	Frank G. (sec.)
2	10·5	11	11
3	10	10	11
5	10	10	11·5

Discussion

Haemophilia is a hereditary disease characterized by lack of anti-haemophilic globulin in the blood. The disease appears only in the male since it is transmitted as a sex-linked recessive gene contained in the X chromosomes, and remains occult in the female.

The theoretical aspect that mating of a female carrier and a male bleeder would produce a homozygous active female bleeder has been shown by Brinkhous and Graham (1950) in dog experiments, in the case of Israels *et al.*, and in Merskey's paper in which he reviews the related literature and presents a haemophilic family with intermarriages for generations and actual bleeding in females.

Theoretically again it is impossible for a heterozygous female (carrier) to manifest active haemophilia, but that this also may happen is illustrated in the case of Fantl and Margolis, who reported a female bleeder without paternal family history, in the case of Taylor and Biggs, who could not detect any deficiency of the A.H.G. in the parents of their haemophilic female, and in the present case, where the father of the haemophilic female and his family were normal and were not related to his wife's family. It is characteristic that all these females have a mild haemophilia and the same mechanism may be responsible for its appearance.

Fantl and Margolis try to explain this phenomenon by accepting that the female is heterozygous in respect of the gene responsible for her son's severe condition, but in her own case the trait is incompletely recessive. Taylor and Biggs, however, think that the female is a carrier of severe haemophilia and the remaining gene has been modified to produce mild haemophilia, her son having inherited the latter.

This explanation fits well with the present case, although we can accept as well the possibility of the manifestation of the recessive gene by the simultaneously defective gene for factor VII.

Since Alexander, Goldstein, Landwehr, and Cook's (1951) first description of congenital factor VII deficiencies, 33 more cases have been reported up to date, which can be divided into two groups: (*a*) familial (17 cases in six families) and (*b*) congenital, although definite family data for some of these are lacking. These cases are of a definite familial character. All familial cases have normal thromboplastin generation and/or prothrombin consumption with the exception possibly of Wurzel, Roth, and Zubrow's (1954) cases which had slightly abnormal prothrombin consumption. This means that the inherited deficiencies are probably of pure factor VII. The congenital group, with the exception of Hicks (1955), Jürgens (1956), and Jenkins (1954) cases, have an abnormal thromboplastin generation and/or prothrombin consumption, and therefore are considered to have other deficiencies additional to factor VII not yet fully established as separate factors playing a role in blood coagulation.

Telfer, Denson, and Wright's (1956) case is deficient in a factor responsible both for abnormal thromboplastin generation and prolonged prothrombin time (Prower defect).

According to Bachmann *et al.* (1957), Prower defect and Hougie's (1956) Stuart factor are identical; they reported a similar case with abnormal thromboplastin generation and nearly lacking factor VII, calling the deficiency Prower–Stuart.

It is probably Prower–Stuart factor, and not factor VII, which is responsible for the haemorrhagic phenomena in de Vries *et al.'s* (1955) and Stefanovic *et al.'s* (1955) cases with the only mildly impaired prothrombin times and the pathological thromboplastin generation. The patients discussed here, having the same prolongation of prothrombin time, do not bleed probably because the deficiency is of factor VII alone and its level must be very low for the manifestation of bleeding. This does not exclude the appearance of haemorrhagic phenomena in their immediate descendants, having in mind Frick and Hagen (1953) and Long, Letendre, and Colpron's (1955) cases in which the parents of the bleeders were mildly affected but symptomless throughout their lives.

Summary

A family with a condition not previously reported of mild pure factor VII deficiency and haemophilia is presented. A female haemophiliac, apparently in the heterozygous state, is included in this family. The literature on the subject is reviewed.

I wish to thank Dr. A. G. Signy for his continuous help and advice, Dr. R. Biggs for providing me with the pure A.H.G., Mr. K. Prentis for his invaluable technical assistance throughout the investigation, and Dr. H. Burkinshaw, under whose care the patient was admitted, for permission to publish this case.

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THE CLINICAL EVALUATION OF STAPHYLOCOCCAL ANTI-ALPHA HAEMOLYSIN TITRES IN PATIENTS' SERA

BY

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(RECEIVED FOR PUBLICATION APRIL 24, 1958)

The relationship between the production of α haemolysin and the pathogenicity of *Staphylococcus aureus* has long been a matter for discussion. Christie, North, and Parkin (1946) stated that the production of α haemolysin is essential for full pathogenicity, and that coagulase-positive strains which produced no α haemolysin might be regarded as non-pathogenic. Marks (1952) found a close correlation between pathogenicity and the production of α haemolysin. On the other hand, Elek and Levy (1950) and Lack and Wailling (1954) found that α haemolysin was not an absolute criterion of pathogenicity. It was suggested by Lack and Wailling that the discrepancy of results might partly be explained by a difference in sources, and on occasions by an over-growth of non-toxigenic strains in necrotic foci. Rogers (1956) has performed experiments which suggest that certain macro-anions, such as chondroitin sulphate, hyaluronic acid, heparin, and the nucleic acids, may, *in vivo*, inhibit the formation of toxins by the organism. The five cases reviewed in this paper were all of infections by a virulent strain of *Staphylococcus aureus*, phage type 80. An attempt will be made to show that in such cases the staphylococcal anti-α haemolysin titre of the patient's serum is a valuable guide to progress and an aid to the detection of latent infection by *Staphylococcus aureus*.

Methods

All the staphylococci isolated from the five cases mentioned were examined for the "clumping factor" by the slide method (Cadness-Graves, Williams, Harper, and Miles, 1943). Hyaluronidase production was determined by the M.C.P. test (McClean, Rogers, and Williams, 1943). Dr. R. E. O. Williams, of the Central Public Health Laboratory, Colindale, phage-typed the organisms.

The strains were tested for α haemolysin by the method of Gillespie, Devenish, and Cowan (1939). The α haemolysin titres of the patients' sera were estimated using the technique described by Lack and Wailling (1954) as follows:

Serial dilutions of the patient's serum, previously inactivated at 56° C. for 30 minutes, were made in 0.5 ml. volumes in saline (to a dilution of 1 in 32). To each tube was added toxin (S.670 Wellcome Research Laboratories), equivalent to 1 international unit of antitoxin, in 0.5 ml. saline. The tubes were shaken, and allowed to stand at room temperature for 30 minutes, after which 0.1 ml. of a 10% suspension of thrice-washed rabbit cells in saline was added to each tube, and to toxin and serum control tubes. The tubes were incubated at 37° C. for one hour, after which the titre of anti-α haemolysin was read. The titre was taken as the tube exhibiting 50% haemolysis.

Anti-leucocidin titres were kindly obtained for us by Dr. G. P. Gladstone, of Oxford, using the technique described by Gladstone and Heyningen (1957). This is based on the fact that staphylococcal leucocidin produces characteristic changes in a coverglass preparation of living human polymorphonuclear leucocytes.

The L+ dose of leucocidin is first assayed by making up mixtures of a standard antileucocidin, and logarithmically falling dilutions of leucocidin. The mixtures are placed on slides, and a coverglass preparation of living leucocytes inverted on top of each. After sealing with paraffin wax, and incubating at 35° C. for 30 minutes, the preparations are examined on a warm stage with light phase contrast.

The standardized leucocidin is then taken, diluted to a 0.2 L+ dose, and put up against logarithmically falling dilutions (10% steps) of the patient's serum, starting at a dilution of 1/10. The procedure is then similar to that used in assaying the L+ dose.

In addition, 15 cases of superficial infection by staphylococci producing α haemolysin were followed for periods up to 18 months to see whether any rise in the patients' anti-α haemolysin titre was produced.

Results

All the staphylococci from the five cases mentioned belonged to phage type 80, and produced a positive clumping test. They all produced

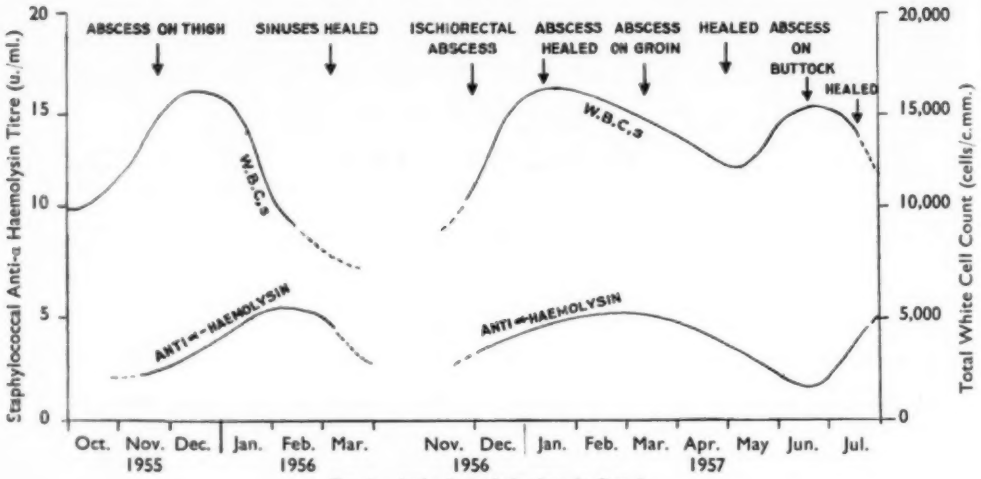

FIG. 1.—Anti-α haemolysin titres in Case 1.

hyaluronidase, although it has been shown that virulent strains may not produce hyaluronidase (Schwabacher, Cunliffe, Williams, and Harper, 1945). Lack (1956) has suggested that hyaluronidase may not act as a "spreading factor" but, by depolymerizing hyaluronic acid in connective tissue, it is removing a potential inhibitor.

All the strains produced α haemolysin. This provoked a rise in anti-α haemolysin titre in four of the cases, but in Case 2 there was no antibody response.

Case 2, a woman aged 68, had chronic osteomyelitis of the right femur from August, 1955, which was still persisting in March, 1958. Although the infecting organism produced α haemolysin, the anti-α haemolysin titre had always been less than 0.5 u./ml. The anti-leucocidin titre was 10 u./ml. This may be regarded as slightly raised, as Dr. Gladstone (personal communication) states that an anti-leucocidin titre of 10 u./ml. or over can be taken as abnormal. However, the anti-leucocidin titre was raised to a much greater extent in the other cases.

In the other four cases, the anti-α haemolysin titre gave a good indication of clinical progress, rising when the infection was most marked and falling on clinical improvement. Fig. 1 shows the variation in anti-α haemolysin titre in Case 1. The maximum anti-α haemolysin titre in this case was 6 u./ml. (An anti-α haemolysin titre of 2 u./ml. or over was regarded as being raised.) The raised anti-α haemolysin titre in Case 1 was of particular value, as the patient presented with several cystic swellings on the scalp, which showed as punched-out defects on radiographs, and a destructive lesion of the left hip. A provisional diagnosis was made of myelomatosis, and it was not until pus was aspirated from the cysts, coupled with the raised anti-α haemolysin titres, that the true diagnosis of pyaemia was made. During an active stage of the patient's disease, when the anti-α haemolysin titre was 4 u./ml., the anti-leucocidin titre was 64 u./ml.

Fig. 2 shows the anti-α haemolysin titre variations in Case 3. This patient had a diffuse, hard swelling of the left thigh, with enlarged inguinal glands. A diagnosis was made of sarcoma, but the anti-α haemolysin titre was 32 u./ml., and the anti-leucocidin titre 100 u./ml. The thigh was therefore incised, and 20 oz. of pus removed. After clinical improvement, the anti-α haemolysin titre had fallen to less than 2 u./ml. and the anti-leucocidin titre to 60 u./ml.

It will be noticed from both graphs that there is a time lag between the appearance of active infection and the elevation of the anti-α haemolysin titre. There is also a delay after clinical improvement before the anti-α haemolysin titre falls to normal, and this may vary between a few weeks and several months.

As can be seen from Figs. 1 and 2, the rise in the total leucocyte count precedes the rise in anti-α haemolysin titre, and the leucocytes fall before the titre. The white cell count may be regarded as a more sensitive index to the momentary condition of the patient. On the other hand, the continued elevation of the anti-α haemolysin titre may indicate a persistence of the infection which is not

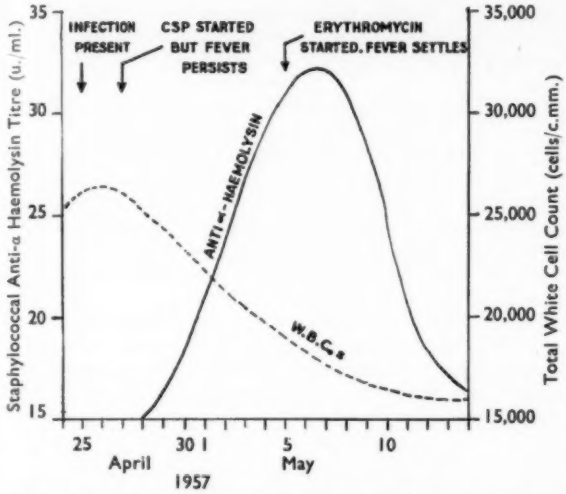

FIG. 2.—Anti-α haemolysin titres in Case 3. CSP = Combined streptomycin and penicillin.

revealed by the white cell count. For instance, it will be seen in Fig. 2 that the leucocyte count was falling towards normal while active infection was still present.

The anti-leucocidin titre bears a close relation to the anti-α haemolysin titre. Fig. 3 shows the anti-leucocidin readings in the four cases, and classifies them according to anti-α haemolysin titres taken at the same time. Case 2 was omitted, as the anti-α haemolysin titre was never raised, and the anti-leucocidin titre only just above normal (Dr. Gladstone has found anti-leucocidin titres of up to 6 u./ml. in normal sera). It appears that an anti-α haemolysin titre of 2 will yield an anti-leucocidin titre of 60 to 70; a titre of 4, an anti-leucocidin titre of 60 to 100; a titre of 16, one of 80 to 100; and a titre of 32, an anti-leucocidin titre of 80 to 125.

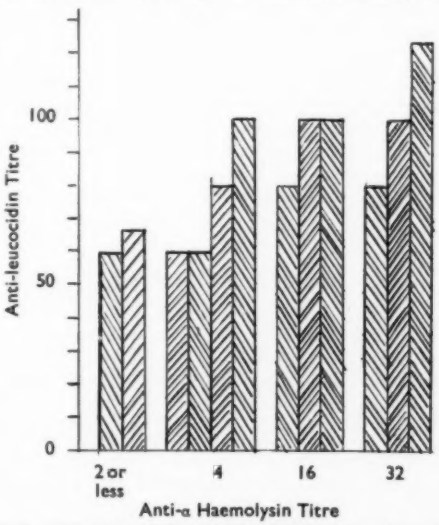

FIG. 3.—Comparison of the anti-α haemolysin and anti-leucocidin titres in the four cases reviewed. Each column represents an anti-leucocidin estimation, an anti-α haemolysin estimation being performed at the same time.

With clinical improvement, the anti-α haemolysin titre may fall to less than 2 u./ml., but the anti-leucocidin titre takes much longer to fall to normal. In Case 5, however, both titres have remained elevated, despite complete clinical improvement. This patient, after having fractured the left femur, developed a large gluteal abscess, staphylococcal pneumonia, and septicaemia. The anti-α haemolysin titre was 32 u./ml. and anti-leucocidin titre 125 u./ml. After a three-month course of erythromycin, the patient was perfectly well, but the anti-α haemolysin titre had fallen only to 16 u./ml. and the anti-leucocidin titre to 37.5 u./ml. Six months later, the anti-α haemolysin titre was <2 u./ml. and anti-leucocidin 60 u./ml.

The amplitude of anti-leucocidin titre variation is greater than that of anti-α haemolysin, and produces a more dramatic picture. For example, during the convalescence of Case 5, the anti-α haemolysin titre fell from 32 to 16 u./ml. whereas the anti-leucocidin titre fell from 125 to 37.5 u./ml. In Case 4, a diabetic woman who died of staphylococcal enteritis, the anti-α haemolysin titre rose only to 4 u./ml., but the anti-leucocidin titre rose to 60 u./ml.

A raised anti-α haemolysin titre has only been obtained when the staphylococci have been situated in muscle, bone, or blood stream. None of the 15 cases with superficial infections by α-haemolysin-producing organisms ever showed a rise in anti-α haemolysin titre in the serum.

Discussion

To-day, when the community is becoming infected with increasing numbers of penicillin-resistant staphylococci, the sooner a diagnosis of staphylococcal infection is made, the better. This is particularly the case with *Staphylococcus aureus*, phage type 80.

It is in the deep-seated infections—in muscle, bone, or blood stream—where isolation of the organism may be impossible or delayed until surgical procedures are taken, that a diagnostic test for active staphylococcal multiplication is of its greatest value. Case 1 was provisionally given the diagnosis of myelomatosis, and the raised anti-α haemolysin titres in Case 3 made it possible to decide on a diagnosis of infection as opposed to one of sarcoma. In latent staphylococcal infections, it must be remembered that the white cell count may be within normal limits. A raised anti-α haemolysin titre, even in the absence of signs of infection, is indicative of the continued presence of the pathogen.

The fact that α-haemolysin antibodies may not be detected in a patient's serum, despite a deep-seated staphylococcal focus, must also be considered. When present at a titre of 2 u./ml. and higher, they are of significance. Leucocidin antibodies are more readily stimulated, and, as exemplified in Case 2, may suggest staphylococcal infection in the absence of a raised anti-α haemolysin titre.

The value of the anti-α haemolysin and anti-leucocidin titres as a diagnostic procedure has been reported by Towers and Gladstone (1958).

The importance of avoiding all delay in the eradication of resistant *Staphylococcus aureus*, phage type 80, from the host is seen by the protracted nature of the illnesses in the cases reviewed. The average duration of infection was two years. Any diagnostic test which can speed the onset of effective treatment for the elimination of the staphylococcus is of value, and the estimation of anti-α haemolysin titres has a definite place in the control of staphylococcal infections.

Summary

Five cases of infection with *Staphylococcus aureus*, phage type 80, have been recorded.

It has been shown that the anti-α haemolysin titre in four of these cases was a good index to the condition of the patient, though less sensitive to the momentary changes of the patient's condition than the total white cell count.

The anti-α haemolysin titre corresponds well with the anti-leucocidin titre.

It is suggested that the anti-α haemolysin titre is of its greatest value in deep-seated infections, when diagnosis may be difficult, or treatment delayed until surgical procedures reveal the causative organism.

We would like to record our thanks to Mr. K. I. Nissen, Mr. Staveley Gough, and Dr. J. S. Richardson for their kind permission to use their cases as a basis for this paper.

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A CRITICAL ASSESSMENT OF THE FREEZE-DRIED DISC TECHNIQUE FOR DETERMINING THE SENSITIVITY OF BACTERIA TO SULPHONAMIDES AND ANTIBIOTICS

BY

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Of the many methods employed for determining the sensitivity of bacteria to antibiotics and sulphonamides, the use of impregnated blotting paper discs has a number of advantages.

It has been pointed out, however, that there are many sources of error in the disc technique, that irregular results are not uncommon, and that the potencies of commercially prepared discs are not always in accordance with their labelled claims (Fairbrother and Martyn, 1951 ; Greenberg, Fitzpatrick, and Branch, 1957).

In view of such criticisms, an assessment of the reproducibility and reliability of the technique carried out in this laboratory was undertaken in order to define the limits of accuracy of routine tests, and to determine the effects of storage on materials employed.

The present investigation shows that discs dried under suitable conditions can be prepared in bulk and stored for relatively long periods without significant loss of potency, and that the amount of test substance per disc can be accurately controlled. Moreover, a high degree of reproducibility of results can be obtained.

Materials and Methods

Preparation of Freeze-dried Discs.—Discs, 5 mm. in diameter, were cut from Ford 428 mill coloured blotting paper, and an arbitrary colour was allocated to each test substance for ease of identification (Bowie and Gould, 1952). After sterilizing, each disc was impregnated with a standard drop (0.02 ml.) of a standard solution of test substance appropriate to the colour of the disc. The discs were then snap freeze-dried in a freeze-drying apparatus (Edwards, Model 3P). Finally, the dried discs were transferred to open-topped glass containers which were stored at +4° C. over anhydrous calcium chloride in outer glass containers fitted with screw caps, and containing a few granules of silica gel to indicate by their colour the prevailing state of desiccation.

Concentrations of Test Substances.—The following concentrations were employed. They are similar to those used by Fairbrother and Martyn (1951).

Penicillin	.. .. 100 units per ml. (2 units per disc)
Aureomycin ..	500 micrommes ,, ,, (10 μg. ,, ,,)
Chloromycetin ..	2,000 ,, ,, ,, (40 μg. ,, ,,)
Streptomycin ..	2,000 ,, ,, ,, (40 μg. ,, ,,)
Terramycin ..	500 ,, ,, ,, (10 μg. ,, ,,)
Erythromycin ..	500 ,, ,, ,, (10 μg. ,, ,,)
Sulphathiazole *..	Saturated aqueous solution at *p*H 8·0 at 37° C. (Approximately 3·2 mg. per ml. and approximately 60 μg. per disc.)

Medium.—Many batches of nutrient media contain a sulphonamide antagonist. This difficulty may be overcome by the incorporation of lysed blood in the medium (Harper and Cawston, 1945 ; Walker, Philip, Smyth, and McLeod, 1947). Petri dishes, 15 cm. in diameter, were employed, containing "oxoid" blood agar base with the addition of 5% defibrinated horse blood previously lysed by freezing and thawing. Care was taken to maintain uniformity in the thickness of the medium in the plates, since variations affect the size of inhibition zones.

Standard Strain of *Staphylococcus pyogenes*.—A strain of *Staphylococcus pyogenes* (N.C.T.C. No. 6571) was used as the test organism throughout.

Size of Inoculum.—The inoculum was standardized to give discrete colonies after 18 hours' incubation at 37° C.

Application of Discs.—Discs were applied by means of sterile forceps to the surface of each agar plate in such a manner that they were evenly distributed, and were firmly adherent. They were sufficiently far apart to prevent synergism and antagonism between antibiotics.

Reading of Plates.—After 18 hours' incubation at 37° C., diameters of zones of inhibition were measured in millimetres and recorded.

* For convenience, sulphathiazole will be included as an antibiotic hereafter.

Experimental Results

Reproducibility of Tests Using Different Batches of Medium.—Experiments were carried out using the methods described above to provide information on the total error of sensitivity testing, and also on some of the components of this error, such as those arising from the variations in media, differences between plates, and differences between the readings made by separate observers.

TABLE I
RESULTS OF STATISTICAL ANALYSIS OF SOURCES OF ERROR

	A	B	C	D	E
Terramycin ..	0·692	5·08	0·286	0·586	2·57
Penicillin	1·314	0·219	0·055	0·522	1·45
Streptomycin ..	0·665	0·055	0·219	0·127	1·03
Aureomycin ..	0·649	1·290	0·312	0·480	1·65
Chloromycetin ..	0·795	0·087	0·246	0·859	1·31
Erythromycin ..	0·111	0·129	0·670	0·296	1·10
Sulphathiazole ..	4·240	0·288	0·560	0·680	2·40

The results of a statistical analysis of these sources of error are summarized in Table I, the methods of analysis being described in the Appendix. (The figures are mean square errors in $mm.^2$, except for column E, which gives the total root mean square error in mm.) The column headings have the following meanings:

A. *Errors in Repetitions of Same Test on Different Days.*—These would arise mainly from variations in the batches of medium and it can be seen that sulphathiazole is outstandingly variable in this respect, as is to be expected owing to the difficulty of neutralizing the sulphonamide inhibitors.

B. *Errors from Differences Between Measurements Recorded by Different Observers on Same Plate.*—Aureomycin and terramycin are particularly affected by this kind of error, suggesting that with each there is a strong subjective element in determining the boundary of the zone of inhibition.

C. *Errors Due to Differences Between Plates Read on Same Day and with Same Batch of Medium.*—All the antibiotics are about equally affected by errors of this kind.

D. *Error Found in Comparing Readings by Same Observer on Repetitions of a Test on Same Plate.*—The accuracy of the assay was about the same for all the antibiotics in this respect.

E. *Total Standard Error of Readings Affected by All Sources of Error in Previous Columns.*—This has been calculated from the square root of the sum of the other entries in the same line. It appears from these figures that all the sensitivity measurements made by the present method are reasonably consistent even when made under the most unfavourable conditions. The range of variations in diameter of the zone of inhibition is from 2 to 5 mm., which, with the sizes of zones considered, represents an error of about 10%.

Possible Effect on Sensitivity Tests of Changes in Strain of Organisms During an Experiment.—Two strains of staphylococci were tested in parallel at intervals over a period of four weeks for their sensitivity to penicillin and sulphathiazole. One strain was the standard strain (N.C.T.C. No. 6571); the other was a relatively resistant strain. One plate was set up for each organism and each antibiotic, and five discs were put on to each plate. The differences between the means of the five diameters on each plate did not vary by more than 1.5 mm. for the penicillin tests, or by more than 2 mm. for sulphathiazole during the course of the experiment, which represents an accuracy of approximately 10%. Readings were made by the same observer throughout, so that one source of inaccuracy was eliminated. The figures given above, however, show that the differences between observers are small for these two antibiotics, and that the introduction of this source of error would not have seriously altered the results.

A more detailed statistical analysis shows that there was no significant variation in the relative sensitivity of the two strains during the period of testing, though there was possibly a tendency for the differences in sulphathiazole sensitivity to diminish as time went on. The values of the differences in diameter of inhibition zones for the two antibiotics are shown in Table II.

TABLE II
DIFFERENCES OF MEAN ZONE DIAMETERS (MM.) BETWEEN A TEST AND STANDARD STRAINS AT INTERVALS FOR FOUR WEEKS

Date	Penicillin	Sulphathiazole
30/5/57	16·6	7·2
5/6/57	16·0	7·6
13/6/57	16·2	6·2
18/6/57	15·6	6·2
25/6/57	16·8	5·8

Effect of Storage on Freeze-dried Discs.—Discs were tested at the beginning and end of batches in routine daily use. The results are summarized in Table III. The second column indicates the time at the beginning and end of which each batch was tested. Apart from sulphathiazole the changes in potency were small. The apparent increase in potency of the sulphathiazole discs was almost

TABLE III

EFFECT OF STORAGE ON FREEZE-DRIED DISCS

Antibiotic	Time (Weeks)	Difference ± Error	% Change	Significance Level
Aureomycin ..	30	−1·60±0·19	−5·8	< 0·001
Terramycin ..	23	−1·56±0·2	−5·6	< 0·001
Chloromycetin ..	25	−0·18±0·31	−0·6	0·60
Streptomycin ..	36	−0·38±0·15	−1·5	0·02–0·01
Sulphathiazole ..	28	+3·48±0·20	+15·6	< 0·001
Penicillin ..	25	−0·42±0·25	−1·4	0·1–0·05
Erythromycin ..	44	−0·70±0·27	−2·3	0·01–0·001

Difference = (mean diameter at first test − mean diameter at second test).

certainly due to the presence of a smaller amount of sulphonamide antagonist in the medium when the second test was carried out.

Relation Between Diameter of Inhibition Zone and Antibiotic Sensitivity in the Disc Technique.—The relationship between the diameter of the zone of inhibition and the actual sensitivity of the organism is not simple. Using the theoretical treatment of the formation of inhibition zones put forward by Humphrey and Lightbown (1952), it is possible to relate the diameter to the sensitivity and provide a rather more satisfactory classification of resistant strains than arbitrary subdivision in terms of diameters. Their analysis suggests that there should be a linear increase in the squares of the zone diameters of a given strain exposed to discs containing logarithmically increasing quantities of antibiotic.

This has been tested on the standard strain for all antibiotics mentioned above except sulphathiazole, and the results are shown in Fig. 1.

The straight line relation is quite closely verified, and, using the values of slopes for the different antibiotics, the expected differences between the diameters of the inhibition zones of the standard strain and others, two, five, and 10 times as resistant were calculated, and are shown in Table IV.

TABLE IV

THEORETICAL DIFFERENCE IN DIAMETER (MM.) OF INHIBITION ZONE BETWEEN A STANDARD SENSITIVE STRAIN AND STRAINS OF 2×, 5×, AND 10× RELATIVE RESISTANCE

Antibiotic	Differences			b
	S=2	5	10	
Aureomycin	2	4	7	100
Chloromycetin	3	8	11	256
Erythromycin	3	7	10	217
Penicillin	3	8	12	269
Streptomycin	2	5	8	144
Terramycin	2	6	9	184

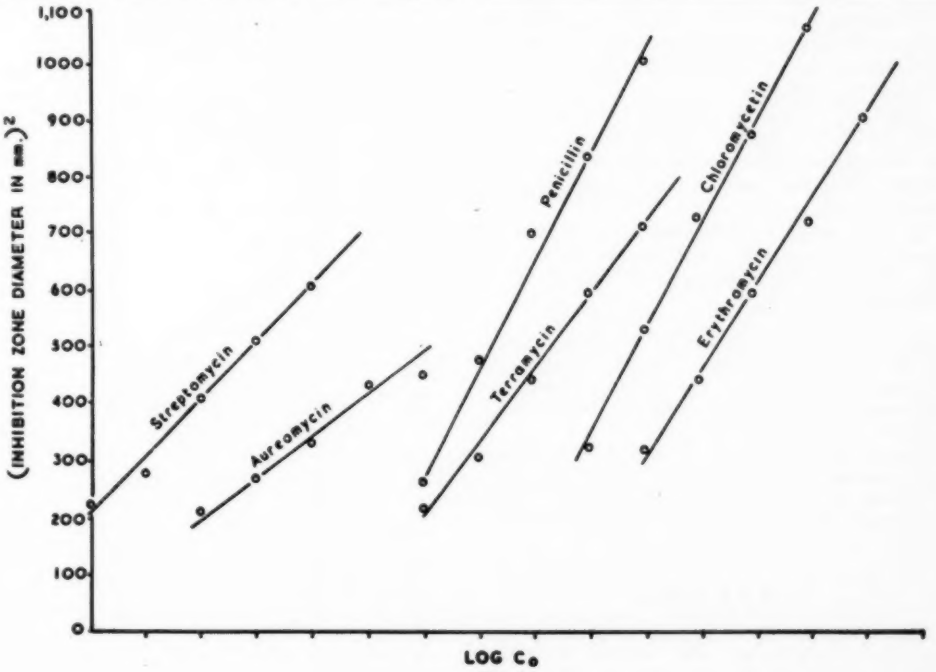

FIG. 1

Referring to Table I column E, it can be seen that a twofold difference in sensitivity is not generally detectable within the limits of error of the test, fivefold is just detectable, and tenfold easily detectable.

On the basis of these experiments, it seems reasonable to classify the resistance of naturally occurring strains according to zone diameters as follows.

(1) Up to five times resistant ..	Sensitive
(2) More than five times resistant but not more than 10 times resistant	Partially sensitive
(3) More than 10 times resistant	Resistant

The diameters of inhibition zones should be determined for the standard strain concurrently with each batch of sensitivity tests, or for each batch of medium.

It is felt that a direct classification in terms of resistance is of more help clinically than one in terms of zone diameter.

Summary

A technique for assessing antibiotic sensitivities using freeze-dried blotting-paper discs is described. This technique has been shown to be highly accurate and reproducible.

Test strains can be quantitatively compared with a standard strain or with one another, and an attempt has been made to relate the diameter of the inhibition zones to the true sensitivity of the organism.

It is suggested that the resistance of naturally occurring strains might be classified on the basis of sensitivity rather than on the diameter of the inhibition zone.

We are most grateful to Dr. S. T. Cowan, Curator of the National Collection of Type Cultures, for granting facilities for the freeze-drying of antibiotic discs, and for providing the strain of *Staphylococcus pyogenes* used as a standard.

Also we thank Dr. R. E. O. Williams, Director of the Staphylococcal Reference Laboratory, for advice during the course of this work, and Mr. P. J. Fisher, who was in charge of the freeze-drying operations.

APPENDIX

The various sources of error in the sensitivity tests were investigated by the usual analysis of variance techniques (see, for example, Snedecor, 1946). The components of variation due to differences between observers and to day-to-day variations in the test were investigated in two separate experiments, and so the interactions between them were not measured. However, the estimates of error arising from the sources given in Table I would not be seriously disturbed by this, and a general figure of about 1 to 2 mm. for the standard error and 4 mm. for the likely range of variation can be applied to all the antibiotics considered. There is, in addition, an error of grouping, since the diameters were read only to the nearest millimetre. A correction for this would reduce the standard deviation of a reading slightly but has not been made since it cannot be incorporated in tests of significance and it seems better to work with an upper rather than a lower limit of error.

The general plan of the tests was to use five plates for each source of variation, e.g., observer or day, and to have five discs on each plate. The final results give the mean square errors for each component and are estimated from the corresponding line in an analysis of variance table. The process is best explained by an example, and for this purpose we may take the experiments on chloromycetin, which are summarized in Table Va and b. The first table gives the results of an experiment in which sensitivity tests were repeated on the same strain by the same observer on different days.

TABLE Va

CHLOROMYCETIN

18/11/55	2/12/55	16/12/55	20/1/56	3/2/56
30	30	31	30	30
30	29	30	30	31
28	29	30	30	32
28	30	32	30	32
31	29	31	30	33
$\bar{x}=29{\cdot}4$	$\bar{x}=29{\cdot}4$	$\bar{x}=30{\cdot}8$	$\bar{x}=30{\cdot}0$	$\bar{x}=31{\cdot}6$
28	30	30	30	32
28	30	30	30	30
30	29	30	30	31
31	29	32	30	32
32	30	30	30	31
$\bar{x}=29{\cdot}8$	$\bar{x}=29{\cdot}6$	$\bar{x}=30{\cdot}4$	$\bar{x}=30{\cdot}0$	$\bar{x}=31{\cdot}2$
28	29	30	31	30
32	30	31	31	32
30	30	32	30	30
30	29	31	31	30
30	30	32	31	31
$\bar{x}=30{\cdot}0$	$\bar{x}=29{\cdot}4$	$\bar{x}=31{\cdot}2$	$\bar{x}=30{\cdot}8$	$\bar{x}=30{\cdot}6$
28	27	32	31	30
30	27	32	31	31
32	27	32	31	31
32	27	31	31	31
28	27	32	30	32
$\bar{x}=30{\cdot}0$	$\bar{x}=27{\cdot}0$	$\bar{x}=31{\cdot}8$	$\bar{x}=30{\cdot}8$	$\bar{x}=31{\cdot}0$
28	30	30	30	31
30	27	30	30	30
32	28	32	31	30
30	27	30	31	30
28	28	30	31	31
$\bar{x}=29{\cdot}6$	$\bar{x}=28{\cdot}0$	$\bar{x}=30{\cdot}4$	$\bar{x}=30{\cdot}6$	$\bar{x}=30{\cdot}4$
$\bar{\bar{x}}=29{\cdot}8$	$\bar{\bar{x}}=29{\cdot}1$	$\bar{\bar{x}}=30{\cdot}9$	$\bar{\bar{x}}=30{\cdot}4$	$\bar{\bar{x}}=30{\cdot}9$

TABLE Vb
CHLOROMYCETIN

Plate	Observers				
	G	*M*	*P*	*T*	*H*
A	30	27	30	28	29
	30	29	30	27	29
	30	29	29	28	29
	30	30	30	27	29
	29	29	31	30	30
	$\bar{x}=29{\cdot}8$	$\bar{x}=28\ 8$	$\bar{x}=30{\cdot}0$	$\bar{x}=28{\cdot}0$	$\bar{x}=29{\cdot}2$
B	31	29	30	29	29
	30	29	31	30	30
	30	31	31	28	30
	30	30	30	30	30
	30	29	31	30	31
	$\bar{x}=30{\cdot}2$	$\bar{x}=29{\cdot}6$	$\bar{x}=30{\cdot}6$	$\bar{x}=29{\cdot}4$	$\bar{x}=30{\cdot}0$
C	31	30	31	32	28
	31	30	30	31	30
	31	31	32	29	30
	31	30	30	30	30
	32	30	30	32	30
	$\bar{x}=31{\cdot}2$	$\bar{x}=30{\cdot}2$	$\bar{x}=30{\cdot}6$	$\bar{x}=30{\cdot}8$	$\bar{x}=29{\cdot}6$
D	30	30	30	32	29
	30	29	32	33	30
	30	30	30	30	30
	30	32	31	32	30
	31	32	30	32	30
	$\bar{x}=30{\cdot}2$	$\bar{x}=30{\cdot}6$	$\bar{x}=30{\cdot}6$	$\bar{x}=31{\cdot}8$	$\bar{x}=29{\cdot}8$
E	29	29	29	32	28
	31	30	29	33	28
	30	29	30	32	27
	30	30	30	29	28
	30	29	29	29	27
	$\bar{x}=30{\cdot}0$	$\bar{x}=29{\cdot}4$	$\bar{x}=29{\cdot}4$	$\bar{x}=31{\cdot}0$	$\bar{x}=27{\cdot}6$
	$\bar{\bar{x}}=30{\cdot}3$	$\bar{\bar{x}}=29{\cdot}7$	$\bar{\bar{x}}=30{\cdot}2$	$\bar{\bar{x}}=30{\cdot}2$	$\bar{\bar{x}}=29{\cdot}2$

The analysis of variance for this experiment is as follows:

	Sum of Squares	D.F.	Mean Square
Between days	88·24	4	22·060
Between plates on the same day	43·76	20	2·188
Residual errors	95·80	100	0·958

The second table gives the results for an experiment in which the same set of five plates was read by different observers. The analysis of variance is:

Between observers	20·288	4	5·072
Between plates	36·688	4	9·672
Observer and plate interaction	46·512	16	2·907
Residual errors	76·000	100	0·760

From the first table the error due to differences between tests made on different days is $(22.060-2.188)/25=0.795$, and that due to differences between plates $(2.188-0.958)/5=0.246$.

The second table gives an estimate of the error arising in readings made by different observers on the same plate. This is calculated as $(5.072-2.907)/25=0.0866$. An approximate estimate of the plate errors can also be made as $(9.672-2.907)/25=0.271$, which compares well with the value 0.246 found in the first experiment. The residual errors, 0.958 and 0.760, are also in reasonable agreement, and do not differ significantly.

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A TRIAL OF RAPPAPORT'S MEDIUM

BY

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In August, 1956, Rappaport, Konforti, and Navon published an account of a new enrichment medium for certain salmonellae. They claimed that their medium was superior for the culture of salmonellae from faeces to selenite F medium and tetrathionate broth except in the case of *Salmonella typhi*.

Materials and Methods

Rappaport's medium was tried out in parallel with, and in combination with, tetrathionate broth, and selenite F medium in 1,000 consecutive stool cultures.

Media.—Selenite F medium, tetrathionate broth, and desoxycholate-citrate-agar (D.C.A.) were made according to the formulae by Mackie and McCartney (1953). Rappaport's medium was prepared according to the formula given by Rappaport *et al.* (1956).

In order to make allowance for the effect of inoculating more bottles, and to test the effect of previous dilution of faeces upon the efficacy of each of the selective media, each specimen was inoculated into all three media both with a small inoculum, that is, 5 drops of a 1:1,000 suspension of faeces in saline, and with a large inoculum, a particle of faeces as big as a pea.

After incubation for approximately 18 hours at 37° C. a loopful from each of the six tubes was plated on to desoxycholate-citrate-agar. After a further overnight period of incubation non-lactose-fermenting colonies were picked off the D.C.A. plates, subcultured on litmus lactose agar to check purity, and when pure investigated biochemically, and, if apparently salmonellae, subjected to serological testing.

TABLE I

COMPARISON OF EFFICIENCY OF DIFFERENT ENRICHMENT MEDIA

	No. of Strains Isolated from Salmonella Groups							
	A	B	C	D	E	F	Others	Total
Selenite F+tetrathionate broth ..	—	2	2	1	7	2	2	16
Selenite F	—	2	3	1	4	2	3	15
Selenite F+tetrathionate broth+Rappaport's medium	—	4	5	2	8	3	4	26

Results

From the 1,000 specimens of stool 31 strains of salmonellae were isolated by one or other enrichment medium or direct plating on D.C.A. The distribution of these according to the medium or combination of media with which they were isolated, and according to groups, is shown in Table I.

Discussion

It is clear from the above results that the addition of a tube of Rappaport's medium to the usual combination of one tube each of selenite F and tetrathionate broth increased the number of isolations of salmonellae from the routine stools examined by about 60% (16 to 26).

It is of some interest to consider how far this increase in the number of strains isolated is due to the specific qualities of the medium, and how far it is accounted for merely by the addition of one further tube irrespective of the medium.

As can be seen from Table I Rappaport's medium used alone (one tube) was superior to single tubes of either selenite F or tetrathionate broth and approximately as efficient as a combination of one tube of each of the two conventional media.

TABLE II

EFFECT OF DIFFERENT COMBINATIONS OF TUBES OF ENRICHMENT MEDIA

	Selenite F	Tetrathionate Broth	Rappaport's Medium	No. of Strains Isolated
Single tube of enrichment medium	+			11
		+		10
			+	15
Two tubes of the same enrichment medium	+			14
		+		14
			+	17
Two tubes one of each enrichment medium	+	+		16
	+		+	18
		+	+	16
Three tubes one of each enrichment medium	+	+	+	26
Four tubes two each of two enrichment media	+	+		21
	+	+	+	24
	+		+	24
Six tubes two of each enrichment medium	+	+	+	29

Table II shows that, while the duplication of tubes of the same medium does increase the number of strains isolated, it was not in the case of tetrathionate and selenite as effective as adding a second tube of the other medium. Two tubes of Rappaport's medium gave almost the same increase in number of strains isolated as the tube of Rappaport's medium and one tube of either of the other two media.

Three tubes, one of each of the three different media, gave a much higher number of strains isolated than any combination of three or even four tubes which involved only two different media.

It is interesting to note that the difference made by using the heavy or the light inoculum (Table III) supported the use of the dilute inoculum for

TABLE III

EFFECT OF INOCULUM SIZE ON NUMBER OF ISOLATIONS WITH THE THREE MEDIA

	Heavy Inoculum (1: 1)	Light Inoculum (1: 1,000)
Selenite	11	10
Tetrathionate	10	8
Rappaport	12	16

(Number of strains isolated.

Rappaport's medium and the heavy inoculum for selenite F and tetrathionate broth, but was very much less than might be expected with the latter two media.

Conclusion

Rappaport's medium is a useful addition to the selective media in use to-day. The addition of one tube of Rappaport's medium to the selenite and tetrathionate in routine use in this laboratory increased the number of isolations by enrichment media from 16 to 26 in 1,000 consecutive unselected stools.

In our hands the sensitivity index of the new medium was not as high as that reported by Rappaport and his colleagues. Taking the "sensitivity index" (S.I.) as the ratio between the number of strains of salmonellae isolated from the population of faecal specimens by the medium under consideration, and the total number of strains isolated from the specimens by any method, in this laboratory the sensitivity index for each medium was as follows: Tetrathionate alone 32%, selenite alone 35%, Rappaport's medium alone 48%, selenite + tetrathionate 52%, selenite + tetrathionate + Rappaport 84%. In the small series reported here there did not appear to be any difference in the efficiency of Rappaport's medium in the isolation of salmonellae of group D, but it did not seem to be as effective as selenite and tetrathionate for the isolation of salmonellae of group E.

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THE INVESTIGATION OF SYMPTOMLESS GLYCOSURIA WITH THE GALACTOSE AND CORTISONE MODIFIED GLUCOSE TOLERANCE TESTS

BY

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Glycosuria is sought in routine clinical examinations in order to detect patients with early or mild diabetes mellitus. There is good evidence that early treatment may avert deterioration and lead to a lower and later incidence of the vascular, renal, and ophthalmic complications (Dunlop, 1954; Ricketts, 1947).

Certain occupations are unsuitable for the diabetic, and early diagnosis is of particular importance in the Royal Air Force because a diagnosis of diabetes is a ban on aircrew duties.

Since other conditions may give rise to glycosuria, its discovery must be followed by a careful investigation in every case to determine the cause. Precision in diagnosis is important in view of the social and economic consequences of a diagnosis of diabetes mellitus, and to provide reliable data for assessing the natural history of the disease and the effects of treatment.

The glucose tolerance test is the most widely used method of determining the significance of symptomless glycosuria. It is based on the fact that after ingesting glucose most patients with frank clinical diabetes have high blood glucose concentrations which are conspicuously abnormal, i.e., they have an impaired tolerance to glucose. By analogy, patients with symptomless glycosuria who have a frankly impaired glucose tolerance when compared with a normal control group are presumed to be diabetic if no other cause for impairment of tolerance can be found. Patients in whom glycosuria is due to a low renal threshold usually exhibit a glucose tolerance which is completely normal. In some cases of glycosuria the glucose tolerance curves are abnormal in certain respects only; in others the curves are abnormal but border on the normal. Controversy exists as to the interpretation to be placed on these curves owing to lack of agreement between authors on the diagnostic criteria to be adopted.

Joslin and Lawrence are two leading authorities who are representative of the two main schools of thought. Joslin, Root, White, and Marble (1952) gave the following criteria for the diagnosis of diabetes mellitus with the 100 g. glucose tolerance test ("true glucose" technique, capillary blood): a fasting blood glucose over 100 mg./100 ml., or a peak glucose value over 170 mg./100 ml. He considered that the two-hour level was "of great value" but that "one cannot disregard the height to which the curve goes. In borderline cases it is well to be conservative and to repeat the test on a later occasion."

On the other hand, Lawrence (1947) attached maximum importance to the two-hour level, irrespective of the peak value of the curve. Using a 50 g. glucose tolerance test (capillary samples and "total reducing substances"), his figures for the upper limits of normal were: fasting 120, peak 200, and two-hour 120 mg./100 ml. Subtracting 15 mg./100 ml. for conversion to "true glucose" values, these figures become fasting 105, peak 185, and two-hour 105 mg./100 ml. He considered that abnormal peak values were of no importance in the presence of a normal two-hour value, and was convinced that diabetes could not be diagnosed without a raised two-hour value. He cited as evidence 80 cases observed by himself (1936) and the fact that normal persons given glucose by duodenal tube and post-gastrectomy cases had abnormally high peaks but normal two-hour values. He attributed these "oxyhyperglycaemic" or "lag" (Maclean, 1922) curves to rapid gastric emptying.

Joslin did not recognize oxyhyperglycaemic curves as constituting a separate group and claimed that he had seen such patients with normal two-hour values whom time had shown to be diabetic.

The present authors felt that although some

"oxyhyperglycaemic" curves were due to rapid absorption of glucose, it was likely on *a priori* grounds that there would be a group in which the same shaped curves were produced by true impairment of glucose tolerance, and that there was a need for further investigation of patients with this type of curve. It was considered that the group of patients with borderline glucose tolerance tests could conveniently be investigated at the same time.

Mosenthal (1947) considered that the intravenous administration of glucose for tolerance testing was advantageous in difficult cases, since irregularities of absorption of glucose from the alimentary tract were avoided. High oral tolerance curves associated with normal intravenous tests do not necessarily indicate rapid intestinal absorption, since Moyer and Womack (1950) demonstrated that the intravenous glucose tolerance test was specific for impairment of glucose tolerance but not very sensitive. There is evidence that the intestinal mucosa may exert an endocrine hyperglycaemic effect during glucose absorption. Laughton and MacCallum (1932) extracted a hyperglycaemia-producing substance from the duodenal mucosa of animals. Loubatières (1954) showed a rise in an alloxan-like substance in the blood after oral administration of glucose; in diabetes a much greater rise occurred. Much less of this substance appeared in the blood of normals and diabetics after intravenous glucose. In view of these observations it was considered that the intravenous glucose tolerance test was unlikely to detect impairment of glucose tolerance of very mild degree and hence would probably be of little value in the diagnosis of patients showing borderline glucose tolerance curves.

Research on the use of galactose tolerance tests in parallel with glucose tolerance tests (Dische, Stamm, and Goudie, 1958) showed that the peak galactose value tended to reflect the rate of absorption of ingested glucose, thus allowing a more specific estimate of glucose tolerance independent of absorption.

Fajans and Conn (1954) described a cortisone-modified glucose tolerance test, which, they believed, might unmask the potential diabetic who had normal glucose tolerance by the conventional test.

In the present investigation an attempt has been made to assess the value of the galactose and the cortisone-modified glucose tolerance tests in the diagnosis of patients with glycosuria showing oxyhyperglycaemic and other types of borderline curves in oral glucose tolerance tests. The results of a follow-up on a small series of oxyhyperglycaemic patients are also reported.

Galactose Tolerance Test

Material and Methods.—Both glucose and galactose tolerance tests have been performed on 65 patients with a history of glycosuria. Eleven were patients requested by us to attend because their previous glucose tolerance tests had shown equivocal results,

TABLE I

DETAILS OF CASES WITH DIABETES, PROBABLE DIABETES, AND PRONOUNCED IMPAIRMENT OF GLUCOSE TOLERANCE

Case Number	Age	% Expected Weight	Family History of Diabetes	Duration of Glycosuria	Symptoms	50 g. Glucose Tolerance Test (mg./100 ml.)			Peak Level Galactose Tolerance Test (mg./100 ml.)
						F.	P.	2	
Group A: Definite Clinical Diabetes									
1	21	91	Neg.	1 month	Polydipsia, polyuria	65*	222	191	32
2	24	102	,,	18 months	,, ,, precoma	87	191	156	54
3	19	93	,,	4 ,,	Polyuria, loss of weight	285	440	404	76
4	36	81	,,	4 ,,	,, polydipsia, rapid loss of weight, ketosis	226	389	320	78
5	22	94	,,	1 month	Ketosis, polyuria, loss of weight	318	492	492	63
Group B: Probable Clinicial Diabetes									
6	39	95	Neg.	2 months	Heart block and ketonuria	108	212	135	42
7	51	112	,,	2 years	Mild thirst and polyuria	122	222	142	43
8	25	92	,,	3 ,,	Loss of 7 lb. in weight in 6 months	179	394	276	86
9	50	127	,,	13 ,,	Recurrent urinary infection	106	252	137	27
Group C: Pronounced Impairment of Glucose Tolerance (peak over 185 mg., two-hour level over 135 mg.)									
10	40	110	Neg.	4 months	None	125	274	184	70
11	44	102	Mother	8 years	,,	160	284	233	25
12	48	94	Neg.	4 months	,,	98	204	157	57
13	36	127	,,	6 years	,,	192	300	266	9
14	44	83	,,	16 ,,	Perspires excessively	72	255	167	130

* Received insulin 24 hours before G.T.T. F. = fasting level; P. = peak level; 2 = two-hour level.

and the remaining 54 were referred for investigation of glycosuria found either during a routine examination or as a result of complaint of symptoms possibly caused by diabetes mellitus. Included in the study were five patients with obvious clinical diabetes, four on whom a diagnosis of "probable clinical diabetes" could be made, and eight who were referred because of glycosuria but who showed no glycosuria during the glucose tolerance test.

On each patient a 50 g. oral glucose tolerance test was carried out using capillary blood and "true" glucose estimations, and on the following day a galactose tolerance test was performed. In each case exactly the same technique was followed as in the normal control series (Dische *et al.*, 1958). Fasting specimens of urine and specimens one and two hours after glucose ingestion were examined by Benedict's qualitative test and the fasting specimen was also examined by Rothera's test. All patients were questioned carefully for present and past symptoms of diabetes. Any past medical history and family history of diabetes were noted. The height and weight were recorded and the weight was also expressed as "percentage of ideal weight" for age, sex, and height from standard insurance tables. A clinical assessment of obesity was also made.

Results.—Tables I, II, and III give a classified list of the most recent glucose tolerance results obtained in the 65 patients with a history of glycosuria investigated, together with their peak galactose values. A brief account of the clinical aspects of each is included.

In order to facilitate comparison between the glucose findings and the galactose tolerance tests, the patients have been classified into groups according to the glucose tolerance results. The normal values mentioned refer throughout to those found in the normal series already reported (Dische *et al.*, 1958).

Group A patients have definite clinical diabetes.

Group B patients probably have clinical diabetes.

Group C patients have no clinical symptoms but pronounced impairment of glucose tolerance with a peak blood glucose level (P) over 185 mg./100 ml. (normal mean + 3 S.D.) and a two-hour value (2) over 135 mg./100 ml. This two-hour value falls within the range of values observed in patients in Groups A and B who can be con-

TABLE II

DETAILS OF CASES WITH MILD IMPAIRMENT OF GLUCOSE TOLERANCE AND OXYHYPERGLYCAEMIA

Case Number	Age	% Expected Weight	Family History of Diabetes	Duration of Glycosuria	Symptoms	50 g. Glucose Tolerance Test (mg./100 ml.)			Peak Level Galactose Tolerance Test (mg./100 ml.)
						F.	P.	2	
Group D: Mild Impairment of Glucose Tolerance (peak over 170 mg., two-hour level 106–135 mg.)									
15	46	103	Neg.	2 years	None	101	190	122	110
16	43	116	,,	4 months	Very doubtful polydipsia and weakness in tropics 1 year before glycosuria	100	212	134	10
17	24	86	,,	2 years	Recurrent boils	116	208	133	71
18	19	99	Grandmother	3 ,,	None	99	224	127	76
19	20	114	Neg.	18 months	Glycosuria found during attack of intussusception	68	195	126	41
20	42	117	,,	4 ,,	None	80	190	108	52
21	19	103	,,	9 ,,	Enuresis, fainting attacks	106	176	128	112
22	23	88	,,	1 month	? Polyuria	103	225	108	104
Group E: Oxyhyperglycaemia (peak over 170 mg., two-hour level 80–105 mg.)									
23	32	99	Neg.	3 years	None. Trace of ketonuria on two occasions	60	187	82	104
24	34	125	,,	1 month	None	90	194	82	53
25	34	120	,,	18 months	,,	82	178	80	70
26	60	109	,,	20 years	,,	98	210	100	73
27	38	125	,,	1 month	Recurrent boils	103	191	105	30
28	19	96	,,	11 months	Listlessness	87	191	97	65
29	36	91	,,	1 month	None	104	198	83	81
30	35	107	,,	1 year	,,	97	180	87	81
31	36	—	,,	5 months	,,	101	194	105	101
Group F: Oxyhyperglycaemia (peak over 170 mg., two-hour level under 80 mg.)									
32	48	114	Neg.	6 months	Presentation—recurrent boils	91	200	66	90
33	38	90	,,	1 month	None	88	199	59	99
34	19	104	,,	2 months	,,	82	184	65	140
35	39	95	,,	10 years	,,	104	179	75	120
36	43	138	,,	10 ,,	,,	102	192	72	82
37	30	93	,,	7 months	,,	91	174	66	115
38	29	116	Mother	1 month	None. Does not appear obese	101	174	52	96
39	36	78	Neg.	2½ years	None. Cholesterol 312 mg./100 ml. Liver function tests normal	93	257	48	164
40	18	101	,,	4 months	None	85	175	73	68
41	21	101	,,	1 month	,,	82	176	52	92

F. = fasting level; P. = peak level; 2 = two-hour level.

TABLE III

DETAILS OF CASES WITH RENAL GLYCOSURIA, AND CASES REFERRED WITH GLYCOSURIA BUT IN WHICH NO GLYCOSURIA WAS FOUND DURING GLUCOSE TOLERANCE TEST

Case Number	Age	% Expected Weight	Family History of Diabetes	Duration of Glycosuria	Symptoms	50 g. Glucose Tolerance Test (mg./100 ml.)			Peak Level Galactose Tolerance Test (mg./100 ml.)
						F.	P.	2	
Group G: Renal Glycosuria (peak level under 170 mg., two-hour level under 105 mg./100 ml.)									
42	39	117	Neg.	10 years	None	91	170	70	68
43	21	94	? Father	1 month	,,	77	140	72	158
44	20	113	Neg.	4 months	,,	86	137	92	49
45	18	100	Brother	5 ,,	,,	83	108	85	11
46	25	91	Neg.	4 years	,,	70	145	68	50
47	19	102	,,	4 months	,,	85	156	95	85
48	19	110	,,	1 year	,,	84	127	98	28
49	32	118	,,	1 ,,	,,	91	164	88	75
50	20	110	Aunt	1 month	,,	83	147	82	15
51	19	—	Neg.	2 years	,,	71	156	49	80
52	21	93	,,	1 month	,,	84	155	76	83
53	18	91	,,	1 ,,	? Polyuria	103	150	78	86
54	31	86	,,	4 years	? Hypoglycaemic attacks	85	146	47	94
55	43	—	,,	13 ,,	None	79	145	69	54
Group H: Renal Glycosuria (two-hour level over 105 mg./100 ml.)									
56	25	116	Neg.	1 month	None	72	149	116	59
57	19	104	? Father, brother, grandfather	1 ,,	None. Acetone reported in urine on one occasion at another laboratory	75	146	137	66
Group J: Investigation for Glycosuria but no Glycosuria Found									
58	33	95	Neg.	1 year	Very doubtful polyuria	111	169	95	94
59	18	106	,,	1 month	Frequency of micturition	91	140	90	20
60	36	113	,,	1 ,,	Recurrent boils	91	136	92	25
61	23	112	,,	1 ,,	None	77	138	57	8
62	19	100	,,	1 ,,	,,	70	138	103	55
63	27	95	,,	1 ,,	,,	90	134	50	78
64	21	98	,,	1 ,,	,,	78	106	100	20
65	50	117	Grandmother		Boils and carbuncles	95	175	71	38

F. = fasting level; P. = peak level; 2 = two-hour level.

sidered to have clinically significant impairment of glucose tolerance.

Group D patients have mild impairment of glucose tolerance with a peak value over 170 mg./100 ml. (normal mean + 2 S.D.) and a two-hour blood glucose level of between 106 mg./100 ml. (normal mean + 2 S.D.) and 135 mg./100 ml.

Group E patients have a peak over 170 mg./100 ml., and a two-hour level of 80 to 105 mg./100 ml.

Group F patients have a peak over 170 mg./100 ml., and a two-hour level under 80 mg./100 ml.

These two groups (E and F) both correspond roughly to Maclean's "lag" or Lawrence's "oxyhyperglycaemic" curves (Maclean, 1922; Lawrence, 1936). The subdivision of cases with abnormal peak values and normal two-hour values into two groups (two-hour level above and below the normal mean of 80 mg./100 ml.) is based on the authors' observation of the inverse relationship of peak and two-hour levels of blood glucose in the normal control series (Dische *et al.*, 1958).

Group G patients have "renal" glycosuria with a peak glucose value under 170 mg./100 ml. and a two-hour value under 105 mg./100 ml.

Group H patients have "renal" glycosuria (peak under 170 mg./100 ml.) but an abnormal two-hour value (105 mg./100 ml. or more).

Group J patients are those who were referred because of glycosuria but had no glycosuria at the time of the glucose tolerance test.

Table IV gives mean galactose peak results and Fig. 1 shows the frequency distribution of galactose peak levels in the different groups, classified according to the glucose tolerance test. There is

TABLE IV

MEAN GALACTOSE PEAK LEVELS IN GROUPS OF PATIENTS STUDIED

Group (see Tables I, II, and III)	Number of Cases	Mean (mg./100 ml.)	S.E. of Mean
A	5	60·6	14·4
B	4	49·5	16·1
C	5	58·2	14·4
D	8	72·0	11·4
E	9	73·1	10·7
F	10	106·6	10·2
G	14	66·9	8·6
H	2	62·5	22·8
J	8	42·2	11·4
Normal controls (Dische *et al*, 1958)	37	65·0	4·9
Post-gastrectomy group (Dische *et al.*, 1958)	7	114·0	4·3

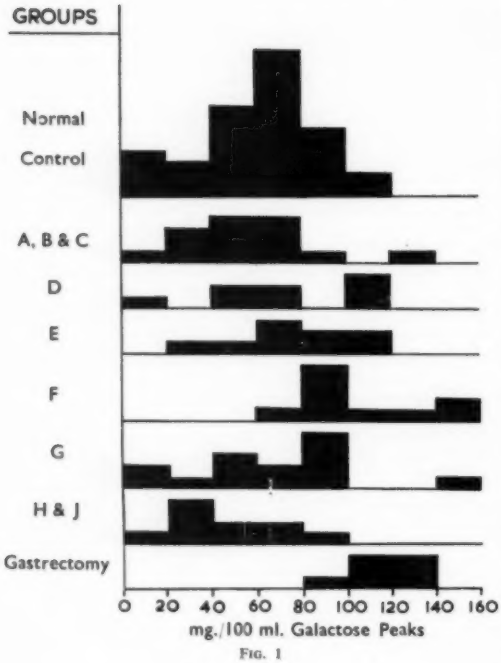

FIG. 1

no significant difference between the peak galactose values obtained in the normal control series and those in Groups A, B, C, D, E, G, H, and J, but a highly significant difference ($P<0.001$) exists between normals and Group F, this being similar to that between normals and the post-gastrectomy patients reported in a previous paper (Dische *et al.*, 1958). There is no significant difference between galactose peak values in Group F and in post-gastrectomy cases.

The possibility that a dose-body-weight relationship might affect galactose tolerance results has been discussed previously and considered an unlikely contingency (Dische *et al.*, 1958). In this series the question does not in fact arise since the mean percentage of expected weight of patients in Group F was 103, while that in the normal group was 102.

Cortisone Modified Glucose Tolerance Test (C.G.T.T.)

The technique of Fajans and Conn (1954) has been applied in a series of 27 patients with glycosuria, five of whom had a family history of diabetes, and in 21 normal controls with no such family history.

Method.—After three days on a standard diet the fasting patient was given 1.75 g. of glucose per kg. ideal body weight as a "base line" test. The next morning a second glucose tolerance test was performed in identical manner, but the patient had received priming doses of cortisone. Subjects weighing less than 160 lb. were given 50 mg. of cortisone acetate orally eight and a half hours and two hours before the ingestion of glucose; those weighing more than 160 lb. were given 62.5 mg. cortisone acetate at the same time intervals. The original technique was followed with the following differences.

(1) Venous blood glucose was estimated in duplicate by a modification of the Folin and Wu method (Dische, 1955) instead of by Somogyi's method.

(2) The glucose was dissolved in 600 ml. of water instead of 400 ml. (Fajans, 1956).

(3) "Cortisyl" (Roussel's brand of cortisone acetate) was used instead of that made by Upjohn Co. and Merck and Co.

Interpretation of Results.—Fajans and Conn regarded patients as definitely non-diabetic if they had a two-hour venous blood glucose level under 110 mg./100 ml. in the base line test; those with a two-hour level over 120 mg./100 ml. were considered diabetic, and those with a two-hour level of 110 to 120 mg./100 ml. as "probably diabetic." In the present series a two-hour level of 115 mg./100 ml. or more in the base line test has been assumed to indicate probable diabetes (mean + 3 S.D. of two-hour levels in normal control base line tests).

Fajans and Conn considered the response to cortisone to be positive in patients with a normal base line test, if the two-hour level in the C.G.T.T. rose to 140 mg./100 ml. or more; from this control series (Table V) a two-hour level of 130 mg./100 ml. or more in the C.G.T.T. of non-diabetics has been adopted as indicating a positive response (mean + 3 S.D. of two-hour levels in the C.G.T.T. control series).

Criteria for a "positive" cortisone response in diabetes were not given by Fajans and Conn, who were mainly concerned with potential diabetics, i.e., patients with a family history of diabetes, who had normal "base line" glucose tolerance tests. They showed, however, in composite curves that in 12 diabetics the mean two-hour level was 40 mg./100 ml. higher in the C.G.T.T. than in the "base line" test and this has been adopted as the criterion of a positive response in the probable diabetics (diagnosed on the baseline G.T.T. two-hour value). It was not possible to assess the significance of rises of less than 40 mg./100 ml., though it is clear that when no rise occurs the result is negative.

Results.—Table V shows the mean and standard deviation of glucose levels in the base line and cortisone glucose tolerance tests (C.G.T.T.) in 21 normal controls with no family history of diabetes.

TABLE V

COMPARISON OF RESULTS OF 1·75 G./KG. BASE LINE GLUCOSE TOLERANCE AND CORTISONE GLUCOSE TOLERANCE TESTS IN NORMAL SUBJECTS WITH NO FAMILY HISTORY OF DIABETES MELLITUS WITH SERIES OF FAJANS AND CONN

	No. of Cases	Mean Age (years)	Two-Hour Venous Blood Glucose "Base Line" (mg./100 ml.)		Two-Hour Venous Blood Glucose, Cortisone G.T.T. (mg./100 ml.)	
			Mean	S.D.	Mean	S.D.
Present series ..	21	24	73·5	13·7	82·3	15·2
Fajans and Conn	37	28	90	?	103	12·4

The results of the C.G.T.T.s in the 27 glycosurics are shown in Tables VI and VII. Twenty-one of these patients had also taken part in the galactose tolerance investigation, and their code numbers are the same as in Tables I, II, and III. The six patients who did not have a galactose tolerance test have been allotted code numbers followed by the letter "C."

TABLE VI

RESULTS OF BASE LINE AND CORTISONE GLUCOSE TOLERANCE TESTS ON NINE PROBABLE DIABETICS

Case Number	Two-Hour Venous Blood Level (mg. per 100 ml.)		Rise in Two-Hour Level on Cortisone	Assessment of Cortisone Response (Positive = Rise > 40 mg. per 100 ml.)
	Base Line Test	Cortisone G.T.T.		
1C*	115	144	+29	?
2C	164	192	+28	?
9	127	119	−8	Negative
17	152	211	+59	Positive
22	124	145	+21	?
27	122	131	+9	?
31	122	124	+2	?
58	139	132	−7	Negative
65*	126	113	−13	"

* Family history of diabetes.

One of the nine probable diabetics showed a clear-cut positive cortisone response, but in five the rise in the two-hour level after cortisone was negligible or non-existent. Only one of the 18 non-diabetics showed a positive response.

It will be noted that the probable diagnosis based on the venous blood glucose two-hour level following a 1.75 g./kg. oral dose of glucose differs in many cases from that made on the 50 g. oral glucose tolerance test (cf. Tables I, II, and III). This point is further discussed below.

TABLE VII

RESULTS OF BASE LINE AND CORTISONE GLUCOSE TOLERANCE TESTS ON 18 NON-DIABETICS INVESTIGATED FOR GLYCOSURIA

Case Number	Two-Hour Venous Blood Level (mg. per 100 ml.)		Assessment of Cortisone Response (Positive = Two-hr. Level >130 mg. per 100 ml.)
	Base Line Test	Cortisone G.T.T.	
3C	74	99	Negative
4C	99	108	"
5C*	85	92	"
6C*	79	91	"
15	92	83	"
19	106	109	"
21	97	106	"
28	79	104	"
35	105	51	"
36	114	84	"
37	86	87	"
39	84	163	Positive
41	44	82	Negative
42	114	115	"
53	63	57	"
54	65	76	"
55	80	75	"
57*	82	121	"

* Family history of diabetes.

Follow-up Series of Patients with Oxyhyperglycaemia

A postal follow-up of 29 patients with oxyhyperglycaemia who had left the service resulted in 14 replies. One had died from essential hypertension and left heart failure. The others were to their knowledge free from diabetes though the mean period since glycosuria had been found was 10 years. Nevertheless, when a further nine patients whose tolerance curves had been confidently labelled oxyhyperglycaemic and who were still in the Service were retested, it was found that two had pronounced impairment of glucose tolerance (Cases 11 and 66, Table VIII). Case 66 had persistent ketonuria.

TABLE VIII

REPEATED GLUCOSE TOLERANCE TESTS SHOWING RELATIONSHIP BETWEEN OXYHYPERGLYCAEMIA AND IMPAIRMENT OF GLUCOSE TOLERANCE

Case No.	Date of Test	Blood Glucose (mg. %)			Comment
		Fasting	Peak	Two-Hour	
11	Jan., 1949*	71	201	81	
	April, 1955	172	296	236	
	May, 1955	160	284	233	
66	Dec., 1949*	85	195	135	Persistent ketonuria. Liver function tests normal. Weight reduced since April, 1955
	Jan., 1949*	96	217	82	
	April, 1955	240	550	350	
	July, 1955	148	316	251	
8	Jan., 1955	122	340	206	Loss of 7 lb. weight in six months. Low-starch diet Jan.–March, 1955
	Mar., 1955	82	215	105	
	Oct., 1955	179	394	276	
15	Mar., 1954	145	225	178	
	Sept., 1954	95	217	93	Weight reduced since March, 1954
	Jan., 1955	101	190	122	
67	Nov., 1955	94	192	135	Loss of 10 lb. by diet in six months between tests
	May, 1956	92	180	96	

* These figures have been corrected for non-glucose reducing substances by subtracting 15 mg.% from the recorded values.

Further evidence for a potential relationship between oxyhyperglycaemic curves and impaired glucose tolerance is shown by the records of patients 8, 15, and 67 in Table VIII. Each of these patients initially showed pronounced impairment of glucose tolerance, but as a result of restriction of dietary carbohydrate the two-hour values became normal though the peaks remained elevated.

Discussion

On the basis of the most recent glucose tolerance test Joslin would consider 34 of the 48 patients in Groups C to H to be diabetic, whereas Lawrence would make such a diagnosis on only 13. Their disagreement is almost confined to patients in Groups E and F, i.e., the oxyhyperglycaemic groups (40% of the total), since Lawrence regards a normal two-hour level as excluding diabetes whereas Joslin regards an abnormal peak or fasting level as diagnostic in themselves. Although patients in this series are not representative of cases of symptomless glycosuria as a whole, less than a quarter of them were specially selected on account of previous difficulty in diagnosis. It is clear that a more definite evaluation of the significance of the oxyhyperglycaemic curves is of considerable importance.

Thirty-nine patients with glycosuria have had more than one glucose tolerance test, and in all have received a total of 113 tests. Taking different curves from the same patient, Joslin would diagnose as both diabetic and non-diabetic 31% of the patients, and Lawrence 33%. This may be explained partly by errors in glucose estimation, and partly by physiological and pathological variation in the patients, but it emphasizes the difficulty of confident diagnosis of these cases.

The difficulties are further accentuated by the differences in probable diagnosis resulting from classification on the basis of the capillary blood 50 g. oral glucose tolerance test and the venous blood 1.75 mg./kg. oral glucose tolerance test. The effect of increasing the dose of glucose above 30 g. is generally regarded as causing no effect on the peak value reached but to extend slightly the period of hyperglycaemia (Maclean and de Wesselow, 1921; Mosenthal and Barry, 1950). We suspect that the difference in the two-hour value may be considerable, and that this probably accounts for the difference of opinion between Joslin and Lawrence on the value of the two-hour figure in diagnosis. The relationship between the 50 g. and 100 g. glucose tolerance tests is to be the subject of further work.

Lawrence (1947) had never had cause to regret his statement that a raised peak with normal two-hour value was normal glucose tolerance and not diabetes. In our experience we have not seen any such patient develop definite clinical diabetes, but Cases 11, 66, 8, 15, and 67 (Table VIII) seem to confirm our suspicion that patients with a normal two-hour value and high peak form a heterogeneous group, viz., patients with rapid absorption of glucose, patients with mild impairment of glucose tolerance, and patients with absorption which is relatively rapid but within normal range and whose ability to metabolize glucose is relatively poor but within normal range.

The finding in the normal control series (Dische *et al.*, 1958) that an inverse relationship exists between peak and two-hour values appears relevant to the diagnosis of normal glucose tolerance in the presence of raised peak values. When absorption is rapid, the supply of glucose in the alimentary tract must diminish quickly; in the presence of a vigorous homoeostatic mechanism the blood sugar would be expected to fall to levels below the average after a high peak. This is in fact the case in post-gastrectomy patients. The mean two-hour glucose value of seven such patients examined by us was 73 mg./100 ml., the two highest being 80 and 83 mg./100 ml. (Dische *et al.*, 1958). When our patients with oxyhyperglycaemic curves are divided into two groups, it is found that the peak galactose values in Group E (two-hour glucose value 80–105 mg./100 ml.) do not differ significantly from normal, whereas there is a highly significant difference between the galactose peaks in Group F (two-hour glucose value under 80 mg./100 ml.) and those in the normal control series. This evidence suggests that glucose is rapidly absorbed in those patients included in Group F.

Tables I, II, and III show considerable variation in the height of galactose peaks within each group of patients classified according to their glucose tolerance test results, but the rate of absorption of sugar from the alimentary tract is likely to vary from day to day in any individual. For this reason the galactose tolerance test cannot be expected to give completely reliable information about absorption of glucose during the preceding day's test, and the interpretation of the test in individual cases must not be too rigid. It is believed, however, that an oxyhyperglycaemic patient with a high peak in the galactose tolerance test and a two-hour glucose level below 80 mg./100 ml. is much less likely to have impaired glucose tolerance than a patient with an abnormally high peak glucose, high normal two-hour

glucose values (80–105 mg./100 ml.), and a low galactose peak. The latter patient is abnormal in three ways: (*a*) He has an abnormally high peak; (*b*) he does not show the normal inverse relationship between peak and two-hour glucose value; (*c*) he presents no evidence of a tendency to rapid absorption of sugar by the galactose tolerance test. It must be borne in mind that mildly impaired glucose tolerance may be a progressive disease in only a very few cases, and may not, even if continued for many years, cause any adverse effects on the patient's health. A large-scale controlled follow-up is necessary to elucidate this point.

An examination of Tables I–III confirms the widely held belief that where the fasting blood glucose level is definitely raised there is impairment of glucose tolerance, but on the other hand pronounced impairment may be present with a normal fasting blood sugar.

Cases 14 and 39 have most unusual findings in common. The results of repeated glucose tolerance tests on them are shown below.

Case No.	Date	F.	P.	2
14	June, 1939* ..	147	217	—
	Aug., 1939* ..	70	230	132
	May, 1942* ..	80	220	90
	July, 1949* ..	60	140	50
	April, 1955 ..	72	255	167
39	Nov., 1953	97	157	125
	May, 1954	85	173	134
	May, 1955	88	172	91
	Feb., 1956	93	257	48

* Corrected to "true glucose" values by subtracting 15 mg./100 ml. for non-glucose reducing substances.

Both patients were symptomless, had very high galactose peaks (Case 14, 130 mg./100 ml., Case 39, 132 mg./100 ml. in May, 1955, and 164 mg./100 ml. in February, 1956), and were both extraordinarily thin men (83 and 78% expected weight respectively). Clinically neither had thyrotoxicosis. Case 39 had a serum cholesterol level of over 300 mg./100 ml. (estimated three times during one week). His serum alkaline phosphatase, bilirubin, and thymol turbidity were normal. He responded positively to cortisone. These tests were not performed in Case 14. Both were probably suffering from the same defect which is perhaps a disturbance of fat metabolism or latent liver disease. If a disturbance of fat metabolism is the cause, then they may be analogous to Lawrence's remarkable case of lipo-atrophic diabetes (Lawrence, 1946). Their galactose tolerance curves may be high in antithesis to the flat galactose curves observed in the obese (Dische *et al.*, 1958). Unfortunately we have been unable to find "normal controls" comparably under weight.

The results with the glucose tolerance test modified with cortisone are strikingly less dramatic than those described by Fajans and Conn. They found some rise in the two-hour level after cortisone in 32 of their 36 diabetics and in all of their eight "probable diabetics" (Fajans, 1956), whereas only six of our nine probable diabetics showed any rise, and in two of these the rise was negligible. The discrepancy may be due to the fact that only five of all our patients but most of those of Fajans and Conn had a family history of diabetes. A C.G.T.T. was performed on three of our patients with a diagnosis of probable clinical diabetes (Cases 1C, 2C, and 9) and none gave a definite positive response: the result in Case 9 was clearly negative. For this reason the cortisone glucose tolerance test in our hands appears insufficiently sensitive to assess the significance of glycosuria associated with borderline glucose tolerance.

Summary and Conclusions

Sixty-five patients with a history of glycosuria have been examined by the oral glucose and the galactose tolerance tests. A cortisone-modified glucose tolerance test has been performed on 27 glycosuric patients.

The oral glucose tolerance test frequently gives equivocal results in the investigation of cases of symptomless glycosuria. In the presence of a normal fasting blood glucose, both the peak and the two-hour blood glucose level should be used in assessment of tolerance. A high peak associated with a two-hour level which is below the average normal is probably caused by rapid absorption of glucose from the alimentary tract and confirmatory evidence may be obtained with the galactose tolerance test. When the peak is high and the two-hour glucose is within the normal range but above the normal average, impairment of glucose tolerance should be suspected, especially in the presence of normal galactose tolerance.

The cortisone-modified glucose tolerance test of Fajans and Conn has been found of no value in the investigation of this series of cases of symptomless glycosuria.

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CHEMICAL EVALUATION OF HEPATIC FUNCTION IN NIGERIANS

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Most of the tests of liver function normally performed in a routine laboratory depend on quantitative and qualitative changes in the serum proteins. Since it is now well established that the serum protein pattern in Africans differs significantly from that generally accepted as normal for Europeans (Arens and Brock, 1954; Edozien, 1957; Holmes, Stanier, Semambo, and Jones, 1951; Schofield, 1957), it appeared of some importance to investigate the results of these routine tests when applied to healthy Nigerians. Such an investigation would not only provide normal values for the local population but may throw further light on the mechanism of the turbidity and flocculation tests of hepatic function.

Materials

Fasting blood samples were collected from 200 healthy adult Nigerians (160 males and 40 females) between the ages of 15 and 40 years. The sera were separated as soon as possible after collection (one to two hours to allow for clot retraction). Most of the analyses were performed on the same day on which the samples were collected, but occasionally when it was not possible to complete the analyses the specimens were stored in the refrigerator at 4° C., and the investigations completed the following morning.

Analyses were undertaken on each sample of bilirubin (total and direct reacting), alkaline phosphatase, proteins, thymol turbidity and flocculation, zinc sulphate turbidity, cephalin cholesterol flocculation, and colloidal gold flocculation.

Cholesterol (total and ester) was estimated and zone electrophoresis on paper for proteins and lipoproteins was also performed; the detailed consideration of this latter group of analyses will be presented elsewhere.

A bromsulphthalein excretion test was also done on 50 of the fasting subjects.

The serum of 50 Europeans resident in Ibadan, 25 cases of infective hepatitis, and 25 cases of hepatic cirrhosis were also examined by the same methods.

Methods

Bilirubin.—The method of Powell (1944) was used. As the final dilution of the serum was 1 to 10, the buffered methyl red standard of King and Coxon (1950) was employed as standard. Direct bilirubin was read at 10 minutes.

Alkaline Phosphatase.—This was estimated by the method of King and Wootton (1956).

Total Proteins.—The biuret method of Wolfson, Cohn, Calvary, and Ichiba (1948) was chosen.

Paper Electrophoresis.—The method of Flynn and de Mayo (1951) was used. Approximately 0.3 mg. serum proteins/cm. Whatman No. 1 filter paper, i.e., 0.2 to 0.25 ml. serum on a 5 cm. wide paper, was applied. Electrophoresis was for 18 hours at 120 volts. The papers were dried for 30 min. at 105° C., then stained for 10 minutes in 0.75% azocarmine B (Gurr) in 50% methanol + 10% glacial acetic acid and finally washed in successive changes of 10% acetic acid until a clear background was obtained. After drying at 105° C. for 15 minutes the papers were oiled with Shandon clearing oil and scanned in an Eel transmission densitometer. Quantitative estimations were made with an Ott compensating planimeter. A globulin correction coefficient of 1.6 was applied. This factor was derived from electrophoresis as described above of a solution containing known quantities of pure albumin and pure γ globulin prepared by continuous paper electrophoresis of pooled human serum. The same coefficient was applied to all the globulin fractions.

Thymol Turbidity and Flocculation.—The estimation was made according to the method of Maclagan (1944, 1947a, 1947b) and Neefe (1946). Two tubes were prepared for each serum, one used to read the turbidity, the other for the flocculation which was read after 18 hours.

Zinc Sulphate Turbidity.—Kunkel's (1947) method was used. Both the thymol and the zinc sulphate turbidities were read after 30 minutes in a "unicam"

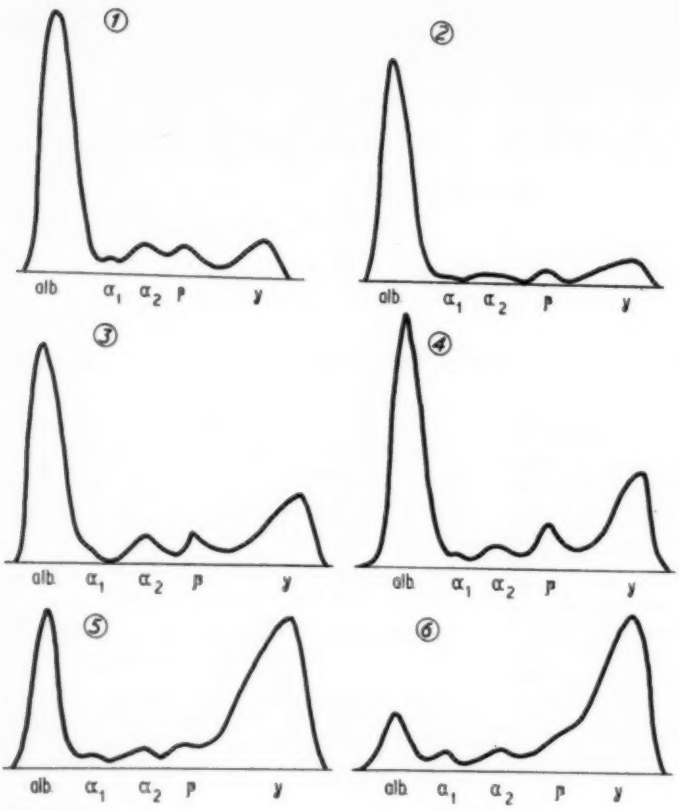

FIG. 1.—Results of electrophoresis. (1) Typical European pattern; (2) Nigerian with low globulin level, < 1.5 g./100 ml. (about 5% of those examined); (3, 4) typical Nigerian patterns; (5, 6) "cirrhosis" of liver in Nigerians.

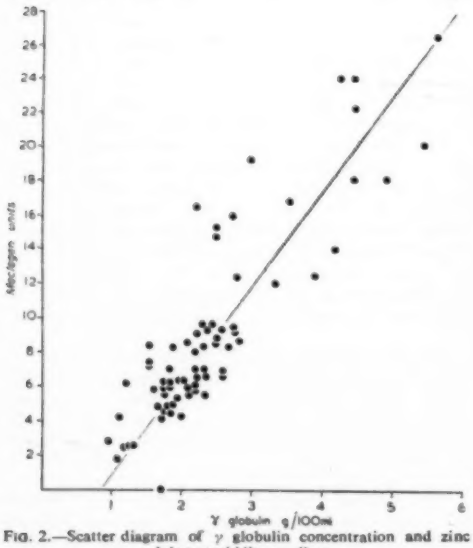

FIG. 2.—Scatter diagram of γ globulin concentration and zinc sulphate turbidity readings.

S.P. 400 spectrophotometer at 660 mμ and the values obtained from a calibration curve. The curve was prepared from diluted serum as described by Varley (1954).

Cephalin-cholesterol Flocculation.—The method was that of Hanger (1939). Commercially obtained cephalin-cholesterol antigen (Difco Laboratories, Detroit, Michigan, U.S.A.) was used. Flocculation was read after 24 hours in the dark. Like other workers we have observed that different batches of cephalin-cholesterol antigen vary greatly in sensitivity.

Colloidal Gold Flocculation.—Maclagan's method (1946) was used with commercially obtained colloidal gold sol (Collosol Aurum, Crookes Laboratories Limited, London).

Bromsulphthalein Test.—This was based on 5 mg./kg. body weight and the method was that of Mateer, Baltz, Marion, and MacMillan (1943). Sterile sulphobromophthalein sodium (Savory & Moore Ltd., London) was employed.

Results

The results are presented in Tables I, II, III, and IV and in Figs. 1 and 2.

TABLE I

RESULTS OF ANALYSIS ON 200 NIGERIANS

Determination	Range	Average
Total bilirubin ..	0·1 to 0·7 mg./100 ml.	
Direct ,, ..	40% to 70% of total	
Alkaline phosphatase	4 to 15 K.-A. units/100 ml.	
Total proteins ..	5·8 to 8·8 g./100 ml.	6·95±0·4 g./100 ml.
Albumin	2·5 ,, 4·5 ,,	3·50±0·3 ,,
Globulin	2·5 ,, 4·5 ,,	3·45±0·45 ,,
A/G ratio ..	0·7 ,, 1·5	1·01
γ Globulin ..	0·9 ,, 2·85 g./100 ml.	2·05±0·35 g./100 ml.
Thymol turbidity ..	0·7 ,, 4·5 Maclagan units	2·10±0·25 Maclagan units
Zinc sulphate turbidity	2·6 to 15 Maclagan units	7·0±1·5 Maclagan units
Thymol flocculation	0 to 1+	
Cephalin-cholesterol flocculation ..	0 ,, 3+	
Colloidal gold flocculation	0 ,, 1+	
B.S.P. retention ..	<5% after 45 minutes	

TABLE II

RESULTS IN ADULT NIGERIANS AND EUROPEANS LIVING IN IBADAN

	200 Nigerians (g./100 ml.)	50 Europeans (g./100 ml.)
Total proteins ..	6·95 ±0·40 (5·8 to 8·80)	6·92 ±0·53 (6·00 to 8·50)
Albumin ..	3·50 ±0·31 (2·5 ,, 4·50)	4·05 ±0·30 (3·50 ,, 5·00)
α_1 Globulin	0·20 ±0·03 (0·09 ,, 0·50)	0·32 ±0·03 (0·21 ,, 0·60)
α_2 ,, ..	0·51 ±0·09 (0·30 ,, 0·80)	0·52 ±0·05 (0·23 ,, 0·80)
β ,, ..	0·69 ±0·15 (0·30 ,, 1·00)	0·83 ±0·12 (0·51 ,, 1·10)
γ ,, ..	2·05 ±0·35 (0·90 ,, 2·85)	1·20 ±0·21 (0·50 ,, 1·60)
Total ,, ..	3·45 ±0·45 (2·50 ,, 4·50)	2·87 ±0·31 (2·00 ,, 4·00)
A/G ratio	1·014±0·18 (0·70 ,, 1·50)	1·411±0·21 (0·80 ,, 1·90)

TABLE III

THYMOL AND ZINC SULPHATE TURBIDITIES IN HEALTH AND IN LIVER DISEASE

Subject	Thymol Turbidity		Zinc Sulphate Turbidity	
	Range	Average	Range	Average
200 Nigerians	0·7 to 4·5	2·1±0·25	2·6 to 15·0	7·0±1·5
50 Europeans	0·5 ,, 4·0	1·9±0·40	0·5 ,, 7·0	3·6±0·9
25 infective hepatitis ..	6·0 ,, 15·0	8·3	9·8 ,, 22·0	16·3
25 hepatic cirrhosis ..	4·4 ,, 10·7	6·9	12·0 ,, 22·5	18·1

Results expressed in Maclagan units.

TABLE IV

FLOCCULATION TESTS IN HEALTH AND DISEASE

Results	Thymol Flocculation				Cephalin-cholesterol Flocculation				Colloidal Gold Flocculation			
	200 Nigerians	50 Europeans	25 Infective Hepatitis	25 Cirrhosis	200 Nigerians	50 Europeans	25 Infective Hepatitis	25 Cirrhosis	200 Nigerians	50 Europeans	25 Infective Hepatitis	25 Cirrhosis
0	185	47	0	1	37	38	0	0	173	46	0	0
1+	14	3	0	0	95	10	0	0	34	3	0	0
2+	1	0	11	8	58	2	6	3	3	1	2	1
3+	0	0	10	12	10	0	8	10	0	0	7	6
4+	0	0	4	4	0	0	11	12	0	0	8	13
5+	—	—	—	—	—	—	—	—	0	0	8	5

Discussion

The serum bilirubin, alkaline phosphatase, and bromsulphthalein retention test give in Nigerians results which are not significantly different from those in Europeans. The serum protein pattern and some of the turbidity and flocculation tests, however, give results which by European "norms" would be considered pathological.

The protein pattern differs in that the serum of Africans contains less albumin and more γ globulin than the serum of Europeans (Fig. 1 and Table II), thus giving lower values for the A/G ratio. The causes of this difference have been the subject of considerable discussion during the last few years. Holmes *et al.* (1951) have suggested that it is related to protein deficiency in the diet, and that the picture can be altered by feeding a high-protein diet. This finding is not in agreement with our observations in Nigerians. Arens and Brock (1954) believe that in Cape Town Africans it is caused by liver damage due to prolonged protein malnutrition, and that in other Africans from areas of endemic tropical disease it is accentuated by chronic parasitic infection. Schofield (1957) considers that it is the result of a pathological process in the liver caused by malarial infection.

The present investigation clearly shows, however, that the high γ globulin levels in Africans are not a result of chronic hepatocellular damage because those tests of hepatic function which may be regarded as fairly specific for liver damage—thymol turbidity, thymol flocculation, and bromsulphthalein excretion (Maclagan, 1948; Mateer *et al.*, 1943)—do not show any abnormality in healthy Africans even though they have a high serum γ globulin level. It also demonstrates that in hepatitis and cirrhosis in Africans there is a further increase in serum γ globulin (Fig. 1) and that the protein flocculation tests show pathological changes similar to those associated with liver disease in Europeans (Tables III and IV).

The most likely explanation seems to be that these differences have a true genetic origin (Edozien, 1957). This view is supported by the finding that a significant difference exists between the zinc sulphate turbidity reading of healthy Negroes and whites in America (Reinhold, 1955). Vera and Roche (1956), working in Caracas,

Venezuela, where malarial infestation is at present a rarity, have also observed that the average γ globulin value in Negroes was higher than in the groups of whites and mestizos (varying mixture of races) who were studied. This work, however, needs confirmation in view of the small numbers of subjects examined. Studies on Nigerians of mixed race are at present in progress. It is possible that residence in the African environment may accentuate this racial characteristic by stimulating an increase in circulating antibodies, but it is important to recognize the fact that the γ globulin in the serum of healthy Africans is normal γ globulin unrelated to hepatic disease.

With regard to the protein flocculation tests, it is evident from Tables I, III, and IV that the thymol turbidity and flocculation and the colloidal gold flocculation tests show no significant differences between healthy Nigerians and Europeans. On the other hand many Nigerians present "abnormal" results with the zinc sulphate turbidity and cephalin-cholesterol flocculation. It is clear, therefore, that the increase in normal γ globulin or the decrease in normal albumin which is observed in most Africans affects the results of the zinc sulphate turbidity and the cephalin-cholesterol flocculation, but does not disturb the thymol turbidity and flocculation and the colloidal gold flocculation. Qualitative changes in albumin and/or γ globulin associated with hepatic disease are reflected in all the tests, including the thymol turbidity and flocculation.

Fig. 2 shows a scatter diagram of the γ globulin values and the zinc sulphate turbidity readings. The results confirm the finding of others (Kunkel, 1947; Discombe, Jones, and Winstanley, 1954) that the zinc sulphate turbidity reflects quantitative changes in serum γ globulin better than the other turbidity and flocculation tests. They also show that correlation between the two is much better with normal sera having γ globulin concentrations less than 3.0 g./100 ml. than with abnormal sera showing higher concentrations of γ globulin.

It is therefore recommended that the best combination of protein flocculation tests for the assessment of hepatic function in Africans consists of thymol turbidity, thymol flocculation, and colloidal gold flocculation. With this combination of tests healthy Africans with or without a high level of serum γ globulin can readily be distinguished from those who have suffered hepatic cell damage. The general impression which has been created that most Africans go through life with damaged livers is unfounded and is undoubtedly due to improper choice of liver function tests. Most investigators in this field have relied on evidence based on A/G ratio, γ globulin estimation, zinc sulphate turbidity, Takata-Ara flocculation, and such other tests which are not sufficiently specific.

Summary

Bilirubin (total and direct reacting), alkaline phosphatase, proteins, thymol turbidity and flocculation, zinc sulphate turbidity, cephalin-cholesterol flocculation and colloidal gold flocculation have been estimated in the serum of 200 healthy adult Nigerians, 50 healthy adult Europeans resident in Ibadan, 25 cases of infective hepatitis, and 25 cases of hepatic cirrhosis.

Evidence is produced to show that the high γ globulin level in the serum of Africans is not due to hepatic cell damage.

It is recommended that the best combination of protein flocculation tests for the assessment of hepatic function in Africans consists of thymol turbidity, thymol flocculation, and colloidal gold flocculation. With this combination of tests healthy Africans with or without a high level of serum γ globulin can readily be distinguished from those who have suffered hepatic cell damage.

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COPPER METABOLISM IN NORMAL ADULTS AND IN CLINICALLY NORMAL RELATIVES OF PATIENTS WITH WILSON'S DISEASE

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Wilson's disease (hepato-lenticular degeneration) is now known to be associated with an inborn error of metabolism. The following biochemical abnormalities have been repeatedly confirmed in patients suffering from the disease (Scheinberg, 1956; Bickel, Neale, and Hall, 1957): Increased content of copper in the liver, brain, and other tissues, increased urinary excretion of copper, a decreased level of copper in the serum and an associated decreased level of the copper-containing plasma protein coeruloplasmin. Other biochemical findings include amino-aciduria without necessarily an abnormal level of amino-acids in the blood.

This familial disease is inherited in a recessive, autosomal way (Bearn, 1953). Biochemical abnormalities have occasionally been reported in clinically normal relatives of patients with Wilson's disease, namely an excess amino-aciduria (Uzman and Hood, 1952) and a low coeruloplasmin level (Scheinberg, Dubin, and Harris, 1955). We are not, however, acquainted with any study of the copper metabolism in asymptomatic relatives, and this investigation was therefore undertaken as a further step in the study of this rare but fatal disease.

As a parallel study in order to establish on a comparable basis the normal range of serum copper levels, one of us has examined the blood of 106 normal adults.

Material and Methods

The serum level and the urinary excretion of copper was estimated in 48 clinically unaffected relatives of cases of hepato-lenticular degeneration. Estimations of coeruloplasmin and urinary amino-acids in the same 48 cases are being carried out by other workers and will be reported later. In all these relatives samples of both blood and urine were examined; 39 individuals in one family were investigated and the family tree (Family A) is shown in Fig. 1. Twenty-three of these were related to the father and 16 to the mother of two patients who died in adolescence of Wilson's disease. Clinical and biochemical studies of these two cases and necropsy findings in one of them have been reported previously (Cases 2 and 8 of Bickel *et al.*, 1957). In addition, the parents and a paternal aunt of two affected siblings in another family (Cases 4 and 11 of Bickel *et al.*) have been investigated (Family B), also the parents of two other proven cases of the disease (Family C), and the elder brother of two affected siblings (Cases 3 and 7 of Bickel *et al.*, Family D).

The control group of normal persons ranged in age from 16 to 54 years and were all members of the hospital medical and ancillary staff.

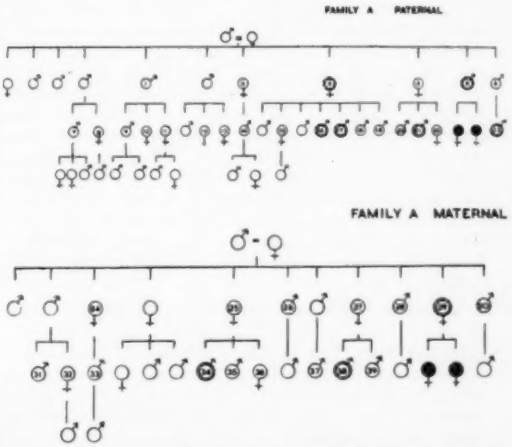

FIG. 1.—Family tree of Family A of whom a total of 39 asymptomatic relatives were investigated. The numbers within the symbols refer to the relatives investigated and can be seen in Table II. ● = the siblings who died of Wilson's disease. The double circles indicate the relatives with a low serum copper level (between 47 and 84 μg./100 ml.).

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Estimation of Copper.—Copper was estimated by a method based on that of Eden and Green (1940) and involves wet ashing of the material. Analyses were carried out in duplicate whenever the sample allowed.

Apparatus.—Special micro-Kjeldahl flasks were made of "pyrex" glass tubing of about 20 mm. outside diameter with bulbs of 35 ml. capacity and graduated at 40 ml. on the stem. They were 9 in. in length and carried a B19 ground-glass joint at the neck; in these flasks both ashing of the sample and extraction of the copper complex was carried out, avoiding the potential sources of error in transference to other apparatus. The importance of clean glassware and pure chemicals cannot be over-emphasized. The special flasks were cleaned initially by boiling in them a mixture of sulphuric and nitric acids, followed by copious washing with tap- and then glass-distilled water. Other glassware was cleaned with chromic acid, washed out with tap water and thoroughly rinsed with glass-distilled water. Polythene vessels were cleaned with concentrated nitric acid before washing.

Reagents.—Reagents used were AnalaR throughout, this grade being found more suitable than M.A.R. chemicals (giving consistently lower blanks). Ammonium citrate, however, is available only in B.P. quality and contains visible dirt contamination and copper in amounts varying from 0.8 to 4.5 parts per million; it is therefore best prepared from AnalaR citric acid and ammonia (201.5 g. citric acid monohydrate requires 158.5 ml. concentrated ammonia solution to give 250 g. ammonium citrate). The 50% solution of this reagent so prepared was further purified as follows:

To a 500 ml. portion in a separating funnel was added a few millilitres each of concentrated ammonia solution and 2% aqueous sodium diethyldithiocarbamate. Amyl alcohol was layered on top and, on shaking, copper diethyldithiocarbamate was extracted from the reagent. This process of extraction was repeated until the top layer no longer contained any trace of yellow coloration, and the lower aqueous layer was then suitable for analysis. Several extractions are needed because removal of the copper complex from this very strong salt solution is relatively slow, whereas it is complete in a single extraction from the weaker salt solution resulting from the ashing process.

Procedure for Serum.—Blood, 25–30 ml., was taken directly into "pyrex" centrifuge tubes (50 ml. capacity) through a dry-sterilized needle made from stainless steel tubing without a mount (the mount is omitted because brass is a potential source of copper contamination). The serum was separated within a few hours of collection.

Five millilitres of serum and two small acid-washed glass beads were put into the micro-Kjeldahl flasks. Concentrated sulphuric acid (2 ml.) and nitric acid (1 ml.) were added and the samples ashed in a Kjeldahl-rack until charred, when a little concentrated nitric acid was added to the flasks (including the blank, consisting only of the acid mixture) until oxidation was complete. Heating must continue for at least an hour after all visible colour has been destroyed in order to avoid yellow-pigmented nitrocompounds when the solution is subsequently made alkaline. Ashing takes two to three hours depending on the amount of organic matter to be destroyed and the frequency of the nitric acid additions. The flasks were allowed to cool, about 5 ml. glass-distilled water was added, and the solution reheated till again fuming to remove nitric acid. When once more cool the contents of the flask were diluted with about 20 ml. glass-distilled water and 50% ammonium citrate (2 ml.) added, followed cautiously with shaking, by concentrated ammonia solution (10 ml.), and, after again cooling, by 2% aqueous sodium diethyldithiocarbamate (0.25 ml.). Distilled water was added to the 40 ml. mark and 5 ml. iso-amyl alcohol added: the flasks were closed with glass stoppers and shaken vigorously for one minute. The top layer was allowed to settle and transferred by means of a teat pipette to a dry, clean test-tube, which was then centrifuged to separate possible suspended water droplets. (Centrifugation was preferred to filtration because varying amounts of contaminating material were found to be extracted from different grades of filter paper.)

The extinction coefficients of the clear yellow solutions of copper diethyldithiocarbamate were measured in a "unicam" quartz spectrophotometer in 1 cm. depth at 435 mμ. Their copper content was obtained by reference to a calibration curve prepared by putting a series of standard solutions containing 0–30 μg. copper through the same extraction process. Under conditions in this laboratory 1 μg. of copper gives an extinction coefficient of 0.044, and the average copper content of the blank was about two-thirds of a microgram.

Stock Copper Standard Solution.—This contains 100 μg. Cu/ml. and is prepared by dissolving 392.8 mg. $CuSO_4.5H_2O$ in 1 litre of distilled water to which a few drops of concentrated sulphuric acid have been added. The stock solution is conveniently diluted 50-fold (2 μg./ml.) for preparation of standards for the calibration curve.

Stability of Colour and Its Proportionality to Copper Concentration.—The extinction coefficients of copper diethyldithiocarbamate solutions in amyl alcohol, prepared as described above, were found to be unchanged after standing overnight at room temperature in glass-stoppered vessels.

Over the concentration range 0–30 μg. copper the calibration curve was found to be completely linear. Samples of greater copper content may sometimes be encountered when ashing tissues and it was found that no loss in accuracy is occasioned by reading these in cells of shorter path length, e.g., 2 mm. instead of 10 mm., or alternatively, by diluting them with amyl alcohol before reading in the 10 mm. cell, since

TABLE I
SERUM COPPER LEVELS IN NORMAL CONTROLS

Male			Female		
Control No.	Age	Copper Level (μg./100 ml.)	Control No.	Age	Copper Level (μg./100 ml.)
1	17	82	54	16	113
2	17	83	55	17	136
3	19	87	56	18	89
4	19	90	57	18	96
5	19	94	58	18	101
6	19	95	59	18	109
7	19	122	60	18	136
8	20	94	61	19	113
9	20	94	62	19	113
10	20	96	63	20	91
11	20	98	64	20	107
12	20	99	65	20	117
13	20	99	66	20	125
14	20	103	67	21	89
15	20	103	68	21	97
16	20	104	69	21	119
17	20	104	70	22	84
18	20	111	71	22	91
19	20	119	72	22	99
20	20	124	73	22	113
21	21	95	74	22	123
22	21	95	75	23	95
23	21	99	76	23	100
24	22	95	77	23	105
25	22	96	78	23	110
26	22	99	79	23	118
27	22	100	80	23	119
28	22	112	81	23	125
29	23	91	82	23	136
30	24	79	83	24	108
31	24	87	84	24	110
32	24	95	85	25	99
33	24	96	86	26	86
34	24	97	87	27	70
35	24	99	88	28	104
36	24	108	89	28	107
37	25	82	90	29	118
38	25	97	91	30	89
39	26	76	92	30	94
40	27	111	93	31	106
41	28	107	94	32	92
42	30	105	95	32	101
43	30	108	96	32	113
44	31	94	97	32	140
45	31	133	98	33	94
46	32	100	99	35	123
47	35	89	100	36	100
48	36	99	101	36	115
49	42	98	102	40	128
50	46	99	103	43	96
51	46	112	104	44	116
52	51	128	105	45	124
53	54	131	106	49	130
Total: 53	Average: 25·4	Mean 100 S.D.±12	Total: 53	Average: 26·2	Mean 108 S.D.±15
Total cases .. 106		Average age 25·8	Combined mean 104 S.D.±14		

equivalent values were obtained if a smaller sample were taken before colour development. This was observed to hold true for samples containing up to 65 μg. copper.

Reproducibility of Results.—This method has been used during the past seven years, and several hundred duplicate estimations have been performed by one of us (F. N.). The maximum variation between duplicates has been 5%, but the great majority of results have shown an agreement within 3%.

Procedure for Urine.—Urine samples were passed directly into polythene bottles and 24-hour collections were made in almost all cases. Since amino-acids were also to be estimated, 5 ml. each of chloroform and glacial acetic acid was used as a preservative. Urinary copper was estimated on samples of the 24-hour collection, but, while 10 ml. is a suitable quantity from a patient with Wilson's disease, rather more is needed from a normal individual. If the volume of urine is too large, calcium phosphate, which should have been sequestered together with iron by the ammonium citrate, may form a troublesome precipitate. Urine, 20 ml., added in two

TABLE II
SERUM COPPER LEVEL AND URINARY COPPER EXCRETION IN 48 ASYMPTOMATIC RELATIVES OF PATIENTS WITH WILSON'S DISEASE

Case No.	Sex	Age	Serum Copper Level (μg./100 ml.)	Urine		Relationship to Patient
				Volume (ml.)	Copper (μg./24 hr.)	
						Paternal
1	M	58	117	620	Nil	Uncle
2	F	56	113	1,400	16	Aunt
3	F	54	82	1,100	15	,,
4	F	50	110	1,350	18	,,
5	M	48	47	2,180	39	Father (3)
6	M	45	100	1,120	25	Uncle
7	M	37	97	2,380	Nil	Cousin
8	F	29	269 (1)	1,230	17	,,
9	M	30	126	1,130	7	,,
10	F	34	113	1,500	31	,,
11	F	28	96	1,020	11	,,
12	F	27	95	1,740	Nil	,,
13	F	25	133	1,460	46	,,
14	M	33	91	1,185	17	,,
15	F	31	165 (2)	1,050	32	,,
16	M	26	71	1,050	21	,,
17	M	23	64	740	22	,,
18	M	18	99	810	13	,,
19	M	12	112	1,110	22	,,
20	M	19	96	1,130	15	,,
21	M	13	84	1,080	5	,,
22	F	9	103	1,000	2	,,
23	M	16	82	1,410	6	,,
						Maternal
24	F	57	109	1,940	14	Aunt
25	F	53	142	1,360	22	,,
26	M	48	118	1,460	20	Uncle
27	F	44	112	1,410	3	Aunt
28	M	42	98	970	2	Uncle
29	F	41	68	2,570	46	Mother (3)
30	M	38	109	900	13	Uncle
31	M	27	118	2,230	9	Cousin
32	F	25	107	1,350	34	,,
33	M	30	132	1,300	23	,,
34	M	27	80	920	33	,,
35	M	22	111	1,890	15	,,
36	F	20	112	1,370	Nil	,,
37	M	15	102	1,060	12	,,
38	M	15	79	1,440	10	,,
39	M	12	108	1,240	5	,,
40	F	61	111	800	25	Mother
41	M	63	90	620	7	Father
42	F	68	127	1,490	7	Pat. Aunt
43	F	40	134	Single specimens	Nil	Mother
44	M	42	93		Nil	Father
45	F	42	147		2	Mother (3)
46	M	44	84		2	Father (3)
47	M	21	72		4	Brother (3)
48	M	5	131		4	Brother
	M 28	Average 29·6	Mean 97 S.D.±20			
	F 20	39·7	Mean 112 S.D.±19			
Total 48		33·8	Mean 103 S.D.±21			

(1) 3 days post-partum } Not included in calculation of mean and
(2) 3 months pregnant } S.D.
(3) Previously reported (Bickel *et al.*, 1957).

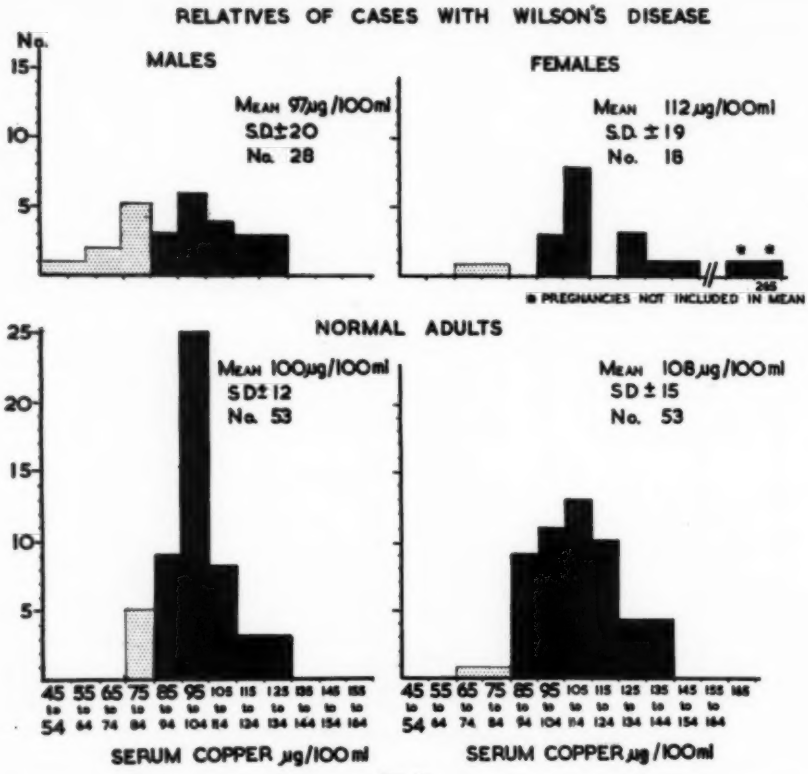

FIG. 2a

FIG. 2.—Distribution of serum copper levels in 53 normal males, 53 normal females, and 48 relatives of patients with Wilson's disease. Values below 85 µg./100 ml. are shown stippled. In previous studies patients with Wilson's disease were consistently found to have levels between 30 and 70 µg./100 ml. The range 70–85 µg./100 ml. is here considered "low normal." A high proportion of these male relatives have a low or "low normal" serum copper level. Nine out of the 28 male relatives have a level below 85 µg./100 ml. as compared with five out of 53 in the control group.

portions of 10 ml., is an adequate sample and guards against occlusion or adsorption losses; the procedure thereafter is as described for serum.

Results

Serum Copper Levels in Normal Controls.—The serum copper levels found in 53 normal males (average age 25.4) and 53 normal females (average age 26.2) are given in Table I and Fig. 2. The male values range from 76 to 133 µg. per 100 ml. with a mean of 100 and a standard deviation of ±12 µg. per 100 ml. The female values range from 70 to 140 µg. per 100 ml., with a mean of 108 and a standard deviation of ±15 µg. per 100 ml. The difference, 8 µg. per 100 ml., between these means is found to be significant ($P<0.01$).

Serum Copper Levels in Relatives of Patients.—The serum copper levels in the 48 clinically healthy relatives are shown in Table II and Fig. 2.

The relatives are numbered 1 to 39 as indicated in the trees of family A. Numbers 40 to 48 inclusive refer to the three other pairs of parents, one paternal aunt and two siblings.

Of these 48 individuals, 11 have a low or normal level (below 85 µg./100 ml.). Two of these are the father and brother (aged 21) of two siblings who died of Wilson's disease. The other nine with low levels (47–84 µg./100 ml.) are all members of one family (family A) of whom a total of 39 asymptomatic relatives were examined. These nine relatives include both parents of the two sisters who died of Wilson's disease, their paterna

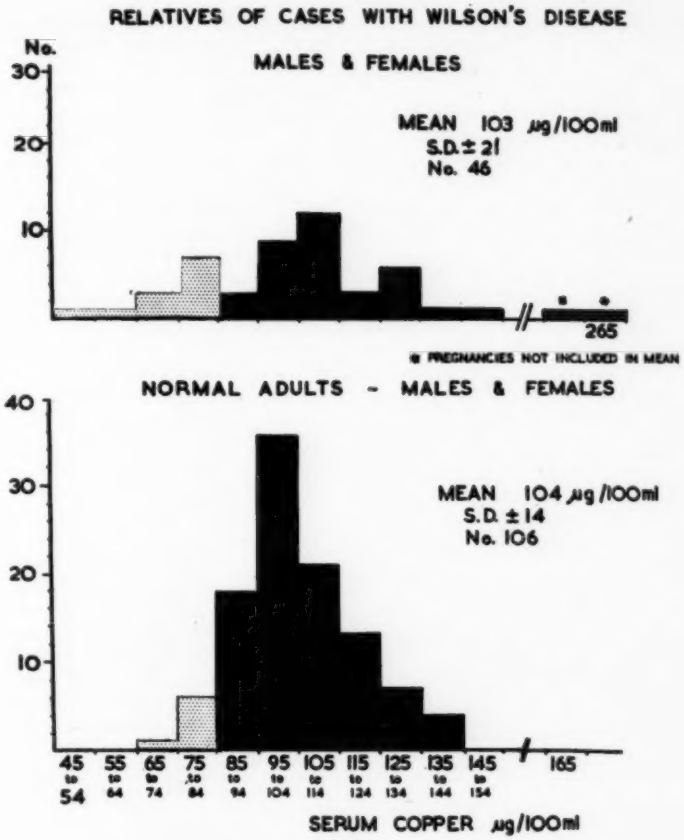

FIG. 2b

See legend on previous page.

aunt, and two of her sons aged 26 and 23, and four other cousins all male (two paternal aged 13 and 16 and two maternal aged 27 and 15).

These low or low-normal copper levels were not found as a " pure streak " in the family. The four male cousins with low levels all respectively had parents with normal copper values. The paternal aunt who had two sons with low levels had two other boys with values well within the normal range. The father was also investigated, as an " internal control," to exclude environmental factors and his values were normal (serum copper 120 µg./100 ml., urinary copper excretion 34 µg./24 hr.).

The abnormally high values found in two relatives were associated with childbearing; No. 15 was three months pregnant and No. 8 had been delivered three days before examination.

The remaining relatives (namely, 35 individuals) have serum copper levels between 90 and 147 µg./100 ml. which falls within our normal range.

Urinary Copper Excretion.—None of the relatives was found to excrete more than 50 µg. copper per 24 hours, the values ranging from nil to 46 µg.

Among 14 cases of Wilson's disease investigated personally, the copper excretion was always high, commonly 200–800 µg./24 hr. in untreated patients, and the lowest value recorded for 24 hours was 100 µg.

The urinary copper excretion in these relatives was therefore normal and well below the pathological level.

Clinico-pathological Data

The first cousin of the mother of the two sisters with Wilson's disease in family A had almost cer-

tainly died from hepato-lenticular degeneration. She had dysphagia and difficulty in walking, and in 1938, when attending the West End Hospital for Nervous Diseases under the care of Dr. Colin Edwards, she showed a "Parkinsonian" mask, mild rigidity with loss of associated movements, and coarse tremor and incoordination of the limbs, particularly the right. She became emaciated and died aged 33. Necropsy performed at the West Middlesex Hospital (P.M. No. 455.38) showed a small nodular liver with advanced cirrhosis and splenomegaly, the spleen being four to five times the normal size, with increased fibrous tissue. There is no record of examination of the brain.

Other deaths in the families confirmed by necropsy had been due to alcoholic delirium tremens (one case), coronary thrombosis (one case), and rheumatic endocarditis (one case).

A medical history was obtained and brief medical examination carried out by one of us on the relatives numbered 1–42 inclusive. A familial incidence of gall-bladder disease (three out of 11 siblings, seven of whom were examined) was found in family A, but this was not considered of significance. A male relative (No. 41) had albuminuria and had been diagnosed the previous month as being in the non-oedematous stage of the nephrotic syndrome. A decreased level of serum copper has been reported in some cases of the nephrotic syndrome (Cartwright, Gubler, and Wintrobe, 1954), but this patient's serum copper concentration was normal (98 μg./100 ml.).

One member (No. 6) suffered from verified ulcerative colitis. No other significant illness was detected.

Bearn (1953) has shown that the incidence of first and second cousin marriages is higher in Wilson's disease than is usually recorded for conditions with recessive inheritance. A high incidence of cousin marriages might reveal other rare recessive abnormalities. In one of our families (Family B) two brothers had married two sisters but there was no known consanguinity.

Discussion

Several points of interest arise out of this study of the copper metabolism of 48 clinically normal relatives of patients with Wilson's disease. First, the serum copper levels in parents of proven cases may be normal or abnormal. Bearn (1953) states that in no parents "available for study was any biochemical abnormality found," but he does not quote the number of cases examined or the copper levels found. Out of the eight parents (four pairs), two (one pair) had abnormally low serum copper levels, the remaining six (three pairs) all being normal (see Table II). Secondly, the criteria for the diagnosis of Wilson's disease need to be considered. Scheinberg (1956) states that "a finding of a level of coeruloplasmin below about 15 mg./100 ml. of plasma, in a subject who is not newly born and is not suffering from the nephrotic syndrome, is probably a sufficient criterion for the diagnosis of Wilson's disease." This cannot be true of the father of one of the proven cases. His serum copper level on three separate occasions over the past 18 months has been 47, 45, and 48 μg./100 ml. respectively. Since 95% of the copper in the serum is firmly bound in coeruloplasmin (Holmberg and Laurell, 1951) and since normally there is 25 mg. of coeruloplasmin per 100 ml. of plasma (Scheinberg and Gitlin, 1952), it is clear that this man has a level of coeruloplasmin well below 15 mg./100 ml. of plasma and yet he is clinically healthy in every respect.

The factors which may influence the serum copper levels have not yet been fully elucidated. Although the level has been found to be relatively steady in both the normal and the abnormal (particularly in the abnormal), some normal controls show unexpected fluctuations. For example, a healthy woman aged 27 (control No. 87) had a level of 70 μg./100 ml. (duplicates 69 and 71 μg./100 ml.) on the first examination, but on five subsequent occasions she was found to have levels ranging from 84 to 91 μg./100 ml. The problem of this fluctuation in normal subjects is at present being studied and the results will be published later.

It has been demonstrated that there is a statistically significant sex difference in that the level is higher in females, which is in accordance with the findings of previous workers in Scandinavia and America (Lahey, Gubler, Cartwright, and Wintrobe, 1953). This higher serum copper level in females may be associated with the higher levels of circulating oestrogens, and the wider range with fluctuating levels of oestrogens. Since the group under discussion mainly included young adults the data cannot be used to correlate possible changes with age. Cartwright *et al.* (1954) in 228 normal adults found that the mean for serum copper was 116±S.D. 14 μg./100 ml. Only 10 of his subjects (4.4%) had a value of less than 90 μg./100 ml.

The occasional low serum copper found in normal relatives of patients with Wilson's disease may be a biochemical abnormality preceding overt clinical manifestations. Alternatively it

may be consistent with a normal span of healthy life. In the latter case it could still represent a hereditary flaw capable of producing disease in a suitably constituted offspring. Since six of the cases are aged 26 to 54 and show no signs of developing hepato-lenticular degeneration, it is most unlikely that biochemical abnormality in their case is going to herald clinical disease. The five others are aged 13–23 and, theoretically, therefore it is possible (although unlikely) that they are candidates for the illness.

In order to decide whether the low serum copper level was fortuitous and unconnected with Wilson's disease or, as seems more likely, of some significance in the inheritance of the disease, it would be interesting to study copper tissue levels in these relatives, but for obvious reasons this is not possible.

Although numbers are small, it may be significant that the low serum copper levels in the relatives were found more frequently amongst the males than the females (see Fig. 2).

Abnormal amino-aciduria was found in five asymptomatic siblings of a family in which four siblings had died of Wilson's disease (Uzman and Hood, 1952). However, Bearn and Kunkel (1954) did not find any excess amino-aciduria in 35 individuals whose siblings suffered from the clinical disease. The present study of copper metabolism in 41 members of the same family shows that this inborn error of metabolism is expressed relatively rarely as a biochemical abnormality dissociated from clinical disease.

Summary

A biochemical study has been undertaken on 48 clinically normal relatives of patients suffering from Wilson's disease.

The copper values obtained in serum and urine in these 48 individuals is reported together with a control study of 106 normal adults.

The urinary copper excretion was normal in all the relatives.

The mean value for serum copper has been found to be 100 μg./100 ml. in normal males and 108 μg./100 ml. in normal females, and this sex difference is statistically significant.

Interpreted in the light of these control values, 35 out of 48 relatives show normal serum copper levels.

Two child-bearing relatives show high values.

The parents of cases of Wilson's disease may be biochemically normal or abnormal.

Eleven asymptomatic relatives showed low serum copper levels and five of these were undoubtedly abnormal (47–70 μg./100 ml.).

Since half of these relatives were beyond the age at which Wilson's disease manifests itself it may be concluded that some unaffected relatives have abnormally low serum copper levels.

We would like to thank Dr. Gilbert Hall for his interest in this study. We also thank all those who co-operated by giving samples of blood, and Drs. G. M. Aber, S. Horner, and E. Price, who obtained the samples from the controls. We are also grateful to Professor Penrose and Dr. Eliot Slater for their interest in the genetical aspects of the work.

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J. clin. Path. (1958), **11**, 448.

THE ESTIMATION OF HIGH CONCENTRATIONS OF PLASMA ACID PHOSPHATASE

BY

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Plasma acid phosphatase estimation is of established value in the diagnosis of carcinoma of the prostate with bone secondaries, and in the assessment of stilboestrol treatment (Gutman and Gutman, 1938; Herbert, 1946; Wray, 1956).

Modifications of the method of Gutman and Gutman (1938), using disodium phenyl phosphate substrate, and estimating the phenol liberated with Folin and Ciocalteu reagent, are widely used. When the result exceeds 40 units/100 ml. it is suggested that the estimation be repeated with diluted plasma (Varley, 1954). Herbert (1946) reduced the time of incubation as an alternative.

Because of the low Michaelis constant for acid phosphatase, approximately 0.0006 (Schønheyder, 1952) and the relatively high concentration of substrate used in the standard technique, a linear relationship between enzyme concentration and activity would be expected over a wider range than the accepted range of 0 to 40 units/100 ml.

The final blue complex produced by phosphotungstic/phosphomolybdic acids, phenol, and alkali, "goes to completion only in the presence of a gross excess of reagent and alkali" (Folin and Ciocalteu, 1927), so that dilution of the final coloured solution with water alone is obviously unsatisfactory. In the present investigation, dilution with reagent instead of with water extended this range of linearity.

Experiments

Reagents.—These are heparinized plasma stored at 2 to 4° C., and a prostatic extract made by grinding a normal prostate in sand with saline. Coarse particles were allowed to settle, and the supernatant fluid diluted in saline.

Methods.—The acid phosphatase activity of serial dilutions of plasma and prostatic extracts was estimated by the method of Gutman and Gutman (1938) as modified by King (1946), employing a final solution volume of 10 ml. to facilitate reading in an EEL photoelectric absorptiometer. When the reading of the test solution exceeded 80, it was diluted 1/5, 1/10, or 1/20 according to the colour intensity. The diluents used were distilled water and, added successively, Folin and Ciocalteu reagent and sodium carbonate and water to maintain the concentration of these reagents. The addition of sodium carbonate was found necessary, for the final concentration must be kept above 3% for maximum colour development. The actual details are as follows:

Addition	Dilution		
	1/5	1/10	1/20
Final coloured solution (ml.)	2·0	1·0	0·5
Folin and Ciocalteu reagent 1/3 (ml.)	1·0	1·1	1·15
Sodium carbonate solution 15% (ml.)	1·6	1·8	1·9
Distilled water (ml.)	5·4	6·1	6·45

After 15 minutes at 37° C. the colour was measured against the blank and standard which had been left at room temperature. Their colour intensity does not change in one hour.

Results

Dilutions of plasma with an acid phosphatase of 720 units/100 ml. from a case of prostatic carcinoma gave similar results to dilutions of prostatic extract. Fig. 1 shows that dilution of the final colour with water was unsatisfactory, for there was no linear relationship between dilution and result (Curve A). By adding Folin and Ciocalteu reagent, together with alkali, linearity was obtained up to approximately 300 units/100 ml.

Discussion

According to the Michaelis and Menten theory, at high substrate concentration the rate of hydrolysis of substrate is proportional to the concentration of enzyme. As substrate is utilized the process finally becomes a first order reaction in which

the rate of hydrolysis is proportional to the concentration of substrate.

Schønheyder (1952), using a continuous titration method, has shown that the kinetics of acid phosphatase do not appear to follow the simple Michaelis and Menten theory, and that phosphate is inhibitory.

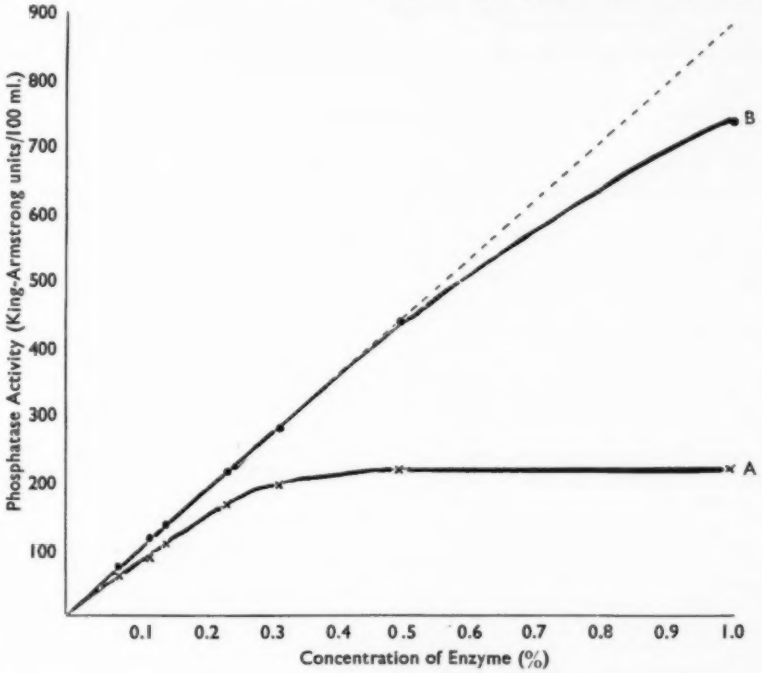

FIG. 1.—Activity/enzyme concentration curves. B=●——●=Final coloured solution diluted with reagent and alkali. A=×——×=Final coloured solution diluted with distilled water.

Using the standard technique of King (1946), and within the limits of routine experimental error, a linear relationship exists between activity and enzyme concentration up to enzyme concentrations of approximately 300 units/100 ml. The limiting factor in this relationship would appear to be colour development by the Folin and Ciocalteu reagent and not inhibition by the products of reaction or exhaustion of substrate, according to the simple Michaelis and Menten theory.

Many causes of misleading high results have been recorded, e.g. (*a*) prostatic massage (Hock and Tessier, 1949) and retention of urine (Wray, 1956), possibly from trauma during catheterization; (*b*) red cell phosphatase liberated by haemolysis during collection may be neutralized by formaldehyde (Abul-Fadl and King, 1948); (*c*) phosphatase of non-prostatic origin may contribute to the result as in Paget's disease (Sullivan, Gutman, and Gutman, 1942), and may be distinguished, by its alcohol resistance, from prostatic phosphatase (Herbert, 1946); (*d*) contamination of the citrate buffer with phosphatase-producing bacteria may also be a cause of error (Herbert, 1946).

The recording of results which might be too low has led to less comment. Failure to recognize the upper limit of colour development reduces the apparent maximum result to the order of 200 units/100 ml. Rapid decay of enzyme activity at 37° C. in neutral solution will introduce a variable error if the plasma is warmed in the water-bath before it is added to the buffer/substrate, in which it is stable.

Summary

Plasma acid phosphatase estimation by the method of Gutman and Gutman (1938, see

King, 1946), using phenyl phosphate substrate and Folin and Ciocalteu reagent, is reliable with enzyme concentrations up to 30 units/100 ml. This limit may be raised to 300 units/100 ml. by dilution of the final coloured solution with Folin and Ciocalteu reagent and sodium carbonate solution. Above this concentration the estimation must be repeated, either with diluted plasma or using a shorter incubation time.

Other causes of error are reviewed.

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AN EXTRACTION TECHNIQUE FOR THE ESTIMATION OF EVANS BLUE IN PLASMA

BY

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The dye-dilution method for the determination of plasma volume presents several problems, one of which is the estimation of dye concentration in plasma. It is with this problem that the present paper is primarily concerned.

The most frequently used dye is Evans blue, T 1824. The colorimetric estimation of this dye in plasma is open to a number of possible errors, particularly those introduced by opacity of the plasma and by the haemolysis of blood samples. Recently it has become possible quantitatively to extract Evans blue from plasma for estimation in watery solution (Allen and Orahovats, 1948; Allen, 1951; Korner, Morris, and Courtice, 1954; Bedwell, Patterson, and Swale, 1955).

As a result of considerable experience with Korner, Morris, and Courtice's method, it has become apparent that certain details of technique are of critical importance. The purpose of this article is to describe a technique that has been found to give satisfactory results, with regard both to speed and to accuracy.

Method

Reagents.—The following are required:

Evans blue, 0.5 g./100 ml., in distilled water (I.C.I.).
Sodium chloride, 0.9 g./100 ml.
Cellulose tissues ("kleenex"), double-thickness sheets of size 21½ × 17 cm.
Teepol L (Shell Chemicals).
Solvent ether B.P.
50% aqueous acetone, made by mixing equal volumes of acetone and distilled water.

Apparatus.—A measure for pulp (capacity 1 ml.) consists of a cylinder of brass rod, hollowed out to a maximum depth of 7/16 in. by a drill of ½ in. diameter having a conical head ¼ in. deep.

Porcelain Gooch crucibles of 8 ml. capacity, with perforated bottoms 1.5 cm. in diameter, and fitted with suitable rubber seals.

A Buchner flask.

Glass filter funnels, each of 2 in. diameter.

Stoppered volumetric flasks of 10 ml. capacity (E-Mil wide-necked flasks were found to be the most suitable).

A spectrophotometer reading at 610 mμ: the instrument used in this work has been the "unicam" spectrophotometer SP 500, with 1 cm. fused glass cells.

Technique.—A 0.5% Evans blue solution in distilled water is injected intravenously in dosage depending on the subject's weight (about 0.075 g./kg.), and samples of venous blood are removed without stasis at suitable intervals. Dry heparin is used as an anticoagulant. The blood samples are centrifuged and the plasma separated.

Teepol, 0.5 ml., is added to 1 ml. of the plasma in a test-tube, and the mixture shaken. It has now to be passed through an adsorption column, which is used in the form of a shallow pad. The pad is prepared in the following manner. A double sheet of "kleenex" tissue, 21½ × 17 cm., is cut into 1–2 cm. squares and placed in a conical flask. Then 27 ml. of 0.9% NaCl is added and the flask shaken until a homogeneous pulp is obtained, leaving about 5 ml. of supernatant saline. Vigorous shaking by hand for five minutes will be found to be adequate. A sufficient quantity of pulp is separated to the neck of the flask and excess fluid allowed to drain from it down the inside of the flask for a few seconds. Small portions of this drained pulp are transferred to the measure of 1 ml. capacity until it is filled levelly to the brim. During the filling of the measure no pressure is used to pack down the pad, but any excess fluid coming to the surface is decanted. The quantity of pulp so obtained is turned out into a perforated porcelain Gooch crucible. The pulp is spread evenly over the bottom of the crucible and tamped down with the end of a glass rod, using only the lightest pressure and paying special attention to the periphery. A well-prepared pad will look homogeneous when the crucible is held up to the light. It is then ready to use.

The plasma-"teepol" mixture is transferred to the prepared crucible (which rests conveniently in a filter funnel) and passes through the pad. It is important that no suction be used at this stage. The first few

drops of filtrate may be used to wash the residue in the test-tube into the crucible, or 0.9% sodium chloride may be used for this purpose. The former technique is convenient, but not necessary, because even the first few drops have been cleared of Evans blue. When no free fluid remains in the crucible it is transferred to the neck of the suction flask and the pad is washed with 5 ml. saline, 5 ml. ether, and then 5 ml. saline, using gentle suction. By now the pad is coloured a pure blue and is ready for the process of elution. This is carried out with about 9 ml.* 50% acetone into a 10 ml. flask, again with the crucible resting in a filter funnel, and then the contents of the flasks are made up to the mark with more of the aqueous acetone. The optical density of this solution of Evans blue is determined spectrophotometrically at a wavelength of 610 mμ, and compared with that of a known standard.

Preparation of the Standard.—About 0.2 g. of the Evans blue from the same ampoule used for the injection is, with an accuracy of 1%, weighed into a 100 ml. flask and made up to the mark with 0.9% sodium chloride solution. Of this solution, 1 ml. is mixed with 0.5 ml. of "teepol" and passed through the same process as the unknown sample. However, unless extreme accuracy is required a simpler technique is adequate, namely, 1 ml. of the 0.5% Evans blue is diluted to 500 ml. in distilled water, and 1 ml. of this solution made up to 10 ml. with 50% acetone and its optical density directly determined. The loss of accuracy caused by not passing the standard through the extraction process is discussed later.

Discussion and Results

Both Allen (1951) and Korner *et al.* (1954) suggest that the dyed plasma should, after mixing with the detergent, be allowed to stand for five minutes. An experiment to determine whether this pause is necessary was performed. Thirty different samples of dyed plasma were estimated, each in duplicate. The spectrophotometer readings of all 60 samples averaged 0.072 (range 0.062–0.083). For one set of samples the five-minute wait was included in the process, but for the duplicate set it was not included. The spectrophotometer readings of the pairs were identical in five pairs, differed by 0.001 in 18 pairs, by 0.002 in five pairs, by 0.003 in one pair, and by 0.005 in the remaining pair. The difference between the means of the two series was 0.0006. It was concluded that the five-minute pause is unnecessary.

With Allen's 1 cm. column, the process of elution takes half an hour or more. Korner *et al.* claim greater rapidity for their technique but give no details of the preparation of their column. The cellulose pad described above gives rapid filtration and elution but adequately prevents loss of unadsorbed dye during filtration. The size of the pad is critical. Too thick a pad greatly slows the procedure of elution, while too thin a pad gives an incomplete extraction. Table I illustrates these two points. It must be stressed that at no stage should water (as opposed to saline or plasma) be allowed to come in contact with the pad, as this alters its texture and reduces its capacity to absorb Evans blue.

TABLE I
EFFECT OF SIZE OF PAD

	Standard (Saline Un-extracted)	Evans Blue Plasma Extracted with Pad of		
		Half Size	Full Size	Double Size
Density	0·054 0·054 0·055	0·049 0·050 0·049 0·050 0·023	0·053 0·051 0·052 0·053 0·052	0·054 0·056 0·054 0·053 0·053
Average duration of elution (min.) ..	—	—	14·2	24·5

The Evans blue used (I.C.I.) has been made up in 10 ml. ampoules by the hospital pharmaceutical department. The absorption spectrum of the preparation has a narrow peak, at a wavelength of 610 mμ. All samples give an adequately linear relationship between absorption and concentration over the range employed, but the exact values differ slightly from batch to batch, and very occasionally from ampoule to ampoule. For this

FIG. 1.—Whether in water, in saline, or extracted from plasma, the light-absorption spectrum of Evans blue is unaffected. ●=Evans blue in water. O=Evans blue in saline. ✕=Evans blue extracted from plasma.

* Direct experiment has shown that Evans blue, in the range of quantities used in this work, is always completely eluted from the pad by 7 ml. of the aqeuous acetone.

reason a direct comparison is always made with a standard from the ampoule used for injection.

The process of adsorption and elution makes little difference to the light-absorption spectrum of the Evans blue, a fact that suggests that the dye remains unchanged in structure (Fig. 1). The Evans blue was not tested for quantity of impurity by the differential elution method of Leeson and Reeve (1949), but their statement that the I.C.I. product is satisfactory from this point of view was accepted in view of the indirect evidence of the absorption curves. Note also that whether the Evans blue is dissolved in water or in 0.9% saline makes no significant difference to the spectrum, or to the density of any given dose of the dye.

Several formal experiments on extraction rates have been carried out. One is summarized in Table II. Three solutions of Evans blue, in water, in 0.9% sodium chloride, and in plasma were

TABLE II
EXTRACTION RATES

Solution (1·5 μg./ml. Evans Blue)	Density	Extraction Rate	
		%A	%B
In water (A)	0·099	—	—
In saline, extracted (B) ..	0·093	94	—
	0·096	97	—
	0·094	95	—
In plasma, extracted	0·094	95	99
	0·095	96	100
	0·094	95	99
	0·092	93	97
	0·090	91	95

made up gravimetrically. One millimetre of the aqueous solution, made up to 10 ml. with 50% acetone, read 0.099. Three 1-ml. portions of the saline solution were passed through the whole procedure and gave readings from 94 to 97% of the unextracted Evans blue. Five 1-ml. portions of the plasma-Evans blue solution, similarly extracted, gave readings varying between 95 and 100% of that given by the extracted saline standard.

It will be noted that the passage of dye through the pad, even in saline solution, results in a definite loss of about 5% of the Evans blue. This loss is about 5% for any concentration of Evans blue in the range employed. Thus Fig. 2 shows the results of an experiment in which two solutions of Evans blue, of strengths 1 μg. and 2 μg. per ml., were extracted from saline and from plasma, and their optical densities compared with those of similar (unextracted) solutions in water.

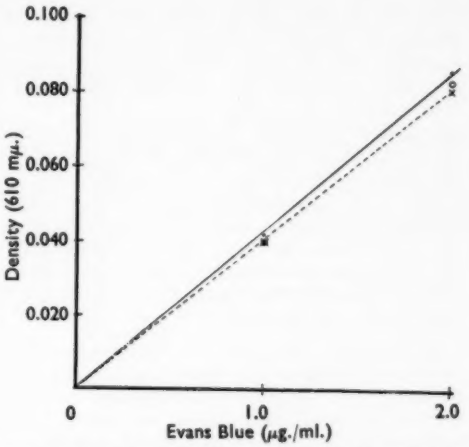

FIG. 2.—A loss of about 5% is incurred in passing dye through the pad, whether in saline or in plasma. •——• Evans blue in water. O - - - - O Evans blue in saline through pad. X - - - X Evans blue in plasma through pad.

However, the extraction rate from plasma, as compared with that from saline, is 95–100% (usually at least 97%). The potential error due to passage through the pad is automatically excluded in the method as described above, by making the standard up in saline and passing it through the pad. Alternatively, if the greatest accuracy is not required, and the aqueous unextracted standard is used, it would be sufficient to subtract 5% from the plasma volume calculated on the basis of this standard. These results were found not to be affected by lipaemia, haemolysis, or excessive bilirubinaemia of the plasma sample.

During the past two years a large number of estimations of Evans blue concentrations in plasma have been made on duplicate samples, and four different operators have been involved. Table III analyses the results of 419 such duplicate

TABLE III
RESULTS IN 419 DUPLICATE ESTIMATIONS

Difference in Spectrophotometer Readings	0·000	0·001	0·002	0·003	0·004	>0·004	
						Explained	Unexplained
No. of samples ..	101	154	92	25	13	15	19

estimations. In 347 cases (83%) the spectrophotometer readings differed by 0.002 or less, i.e., a percentage difference of only 2 to 3%. In a further 38 cases (9%) the difference between the duplicate readings, 0.003–0.004, was still less than

5%. As in this region of its scale the spectrophotometer is calibrated only by 0.005 and finer readings must be made by eye, these results are considered satisfactory. Greater discrepancies were observed in 34 cases (8%), but in 15 of these the causative error of manipulation had already been appreciated or was discovered in immediate retrospect. The remaining 19 were unexplained and must be considered a failure of the method: though, as it has been shown that the usual factors making difficult the estimation of Evans blue in plasma do not affect this method, it is considered likely that here too the errors involved were purely manipulative in nature.

The clinical value of any method for blood volume estimation is in inverse proportion to the time taken to complete the estimation. With the method described here, the estimation of a blood sample for its Evans blue content will be complete by the time a reliable reading of the haematocrit (say 30 minutes for spinning) has been obtained.

Summary

A technique for the estimation of Evans blue in plasma is described. The dye is adsorbed on to a small pad of cellulose pulped in 0.9% sodium chloride, which is then washed with saline, ether, and saline again, and extracted with 50% aqueous acetone.

Evidence is presented for the accuracy of the method, on the basis both of laboratory experiments and of day-to-day experience with the method.

Our thanks are due to Dr. L. MacDonald, and to Dr. S. T. H. H. Pilbeam and Dr. E. T. Knudsen, of the Courtauld Institute of Biochemistry, who did valuable early studies with the technique, and to Mr. L. P. Le Quesne for his kind collaboration throughout.

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J. clin. Path. (1958), **11**, 455.

TECHNICAL METHODS

A Glass Tissue Culture Chamber for Use in Time-lapse Cinematography

F. L. CONSTABLE AND MARGARET A. J. MOFFAT

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(RECEIVED FOR PUBLICATION MARCH 3, 1958)

Numerous tissue culture chambers have been devised with the object of providing the optical requirements necessary for microscopic observation of the culture, and some of these incorporate a means of perfusion to facilitate changing medium, or to introduce various agents during the period of observation (Carrel, 1931; de Haan, 1931, 1938; Lindbergh, 1939; Parker, 1950). A few have been designed specifically for use in microcinematography in conjunction with phase-contrast or interference microscopy in which the optical requirements are exacting and impose strict limitations upon the design (Hu, Holmes, Pomerat, Livingood, and McConnell, 1951; Pomerat, 1951; Christiansen, Danes, Allen, and Leinfelder, 1953; Schwöbel, 1954; Dick, 1955; Richter and Woodward, 1955).

Requirements

The chamber herein described was developed to meet the following general requirements:

(1) The purpose of the chamber is to permit time-lapse colour cinematography with interference microscopy of the growth of normal or virus-infected tissue culture cells.

(2) It is essential that the walls of the chamber be optically flat and parallel and of uniform refractive index. The thickness of the chamber must not exceed 1 mm. and the medium must be homogeneous.

(3) The culture must be maintained for at least three days, and this, in view of the small dimensions of the chamber, necessitated by optical considerations, implies the use of perfusion, which need not be continuous.

In addition the following were considered to be desirable: (*a*) The material to be glass which combines relatively easy working with ease of sterilization; (*b*) the method of perfusion to be as simple as possible, avoiding large reservoirs, complicated tubing and valves; (*c*) the adhesive for sealing the chamber to be more durable than wax, to provide a perfect seal, to be non-toxic and to withstand sterilization at 160° C.; and (*d*) the construction to be within the capabilities of a small laboratory workshop and the cost to be as low as possible.

Construction

The body of the chamber is made from a thin glass microscope slide. 3 in. × 1 in. × 1 mm., a hole approximately ½ in. in diameter being cut in the centre, with a No. 1 coverglass, 0.18 mm. in thickness, above and below to form the walls.

At each side of the perforation a short groove is cut in the glass, parallel to the long axis of the slide. These should be no deeper than is necessary to accommodate a stainless steel hypodermic needle No. 20, which should not project above the surface of the glass within the area of the coverglass seating.

At this stage all components of the chamber are cleaned to the requisite standard for tissue culture.

To assemble the chamber one coverglass is cemented over the hole in the slide on the surface on which there are no grooves; two hypodermic needles are bent into the shape of a crank and are cemented

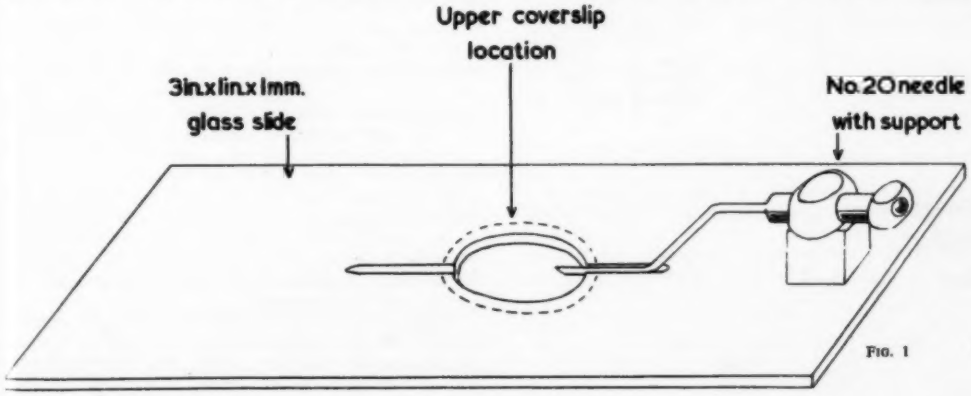

FIG. 1

into the grooves so that the points project slightly into the chamber. Additional support is given in the form of small wooden blocks under the needle fitting to facilitate the attachment of syringes or tubing. Finally, a coverglass is cemented in place to close the chamber, the only access to which is now through the needles (Fig. 1).

A satisfactory cement was found to be "araldite" which is a two-component epoxy resin, obtainable from Aero Research Limited, Duxford, Cambridge. At room temperature this adhesive takes at least 12 hours to set, but by raising the temperature this period can be reduced to as little as 30 minutes.

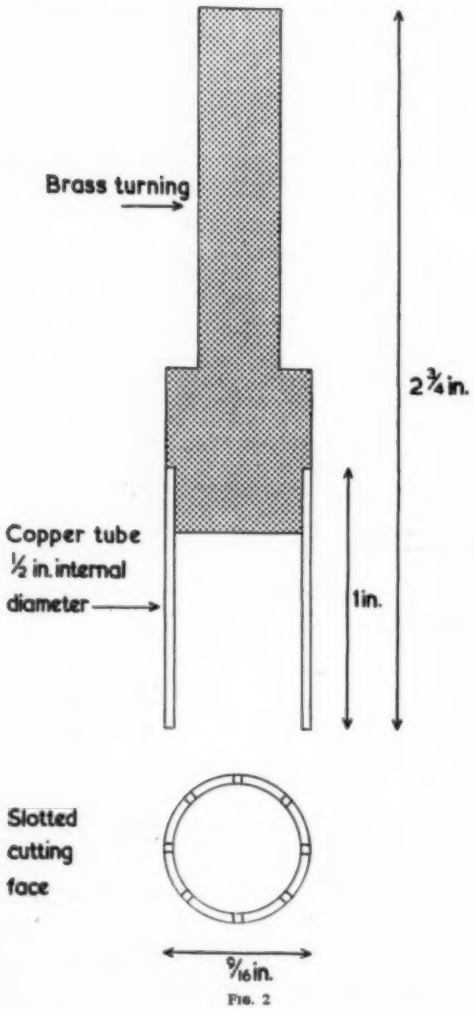

FIG. 2

Cutting the Glass

Holes can readily be cut in glass slides if a power-driven vertical bench drill is available. An effective cutter was made by turning a brass rod to the dimensions shown in Fig. 2, and soldering to it a length of copper tubing. If a lathe is not available, short lengths of tubing of suitable diameter soldered together in telescope fashion will suffice. Slotting the face of the cutting edge with a fine hacksaw increases the efficiency. In operation the tool should be fed with coarse carborundum paste and the drill speed should be fairly low. To prevent cracking, a perfectly flat support must be provided for the slide, and drill pressure carefully regulated, especially towards the end of cutting. A carborundum wheel in a dental drill serves to cut the needle grooves. The operation should take less than five minutes.

Sterilization

The complete chamber is sterilized in the hot air oven at 160° C. for one and a half hours when the cement sets extremely hard and may become black.

The Chamber in Use

Preparation of Cultures.—After sterilization the chamber is filled with a suspension of cells at a density of approximately 100,000 cells/ml. in an appropriate medium. This is injected from a syringe directly attached to one of the needle fittings and by tilting the slide all air can be excluded. After removal of the syringe it is necessary to seal the needle ends, for which small plugs of sterile cotton-wool are satisfactory, to prevent blockage by dried medium. A short period of incubation at 37° C. is required to allow the cells to settle on the upper coverglass, after which the chamber is turned over and is ready for observation in a microscope incubator.

Changing Medium and/or Perfusing Chamber.—This may be accomplished in one of two ways, depending on whether or not the slide can be moved from the microscope stage.

If the slide can be moved the fluid is changed by means of a syringe, as in the initial filling. If, however, it is to remain *in situ*, a method of perfusing the chamber must be provided. For this purpose a reservoir is placed inside the incubator, fluid being drawn into the chamber under negative pressure applied by an externally situated syringe, connexions being made by polythene tubing and needle adaptors.

The syringe may be operated by hand, or for prolonged irrigation, either continuous or intermittent, by a suitably geared motor. To introduce fresh medium or agents such as viruses, it is only necessary to replace the reservoir and this operation can be carried out during filming.

Although the volume of the fluid in the chamber is small it should support cell growth without renewal for as long as 48 hours, and in our experience only one or two manipulations, either to introduce virus or

fresh medium, were found to be necessary during the three-day period of filming.

Discussion

The chamber as constructed and used was found to fulfil all of the requirements. Some difficulty was experienced, however, in carrying out the static perfusion technique, but by exercising patience and by excluding all air bubbles this method of changing the medium is practicable. In addition to using this system to perfuse nutrient fluid for cell growth it may be used as a means of adding any desired infective or chemical agent. As pointed out by Dick (1955), an important advantage of the negative pressure technique is the virtual elimination of the large "dead" space inherent in certain other systems.

The needles are the only metallic components of the chamber, but being of stainless steel are not toxic to the tissue culture. The cement also is not toxic, and if used with discretion need scarcely come into contact with the medium. The heat of sterilization sets the cement so hard that it is impossible to detach the coverslips and although the needles can be recovered the slide must be rejected. A high melting point wax was used at first for one or both coverslips to permit repeated use of the slide, but the glass cutting proved so easy and quick that it was decided to regard the chambers as expendable rather than forgo the advantages of a permanent seal.

Soda glass coverslips, if adequately cleaned and sterilized, were found to support a monolayer of HeLa cells and were optically adequate for interference microscopy. The fluid layer, being thin and homogeneous, does not interfere with colour interpretation and light absorption is minimal.

Summary

An easily made glass tissue culture chamber is described which fulfils the requirements necessary for the observation of normal or infected tissue culture cells by time-lapse colour cinematography. A method of perfusion under negative pressure is incorporated in the design.

We are indebted to Mr. T. C. Dodds, Mr. G. A. Wilson, and Miss Sheila Heath for technical assistance.

One of us (M. M.) was in receipt of an Edinburgh University Graduate Research Scholarship.

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Preparation of Stained Films of Sickle Cells

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(RECEIVED FOR PUBLICATION SEPTEMBER 23, 1957)

It is known that red cells from a patient showing sickle-cell anaemia or sickle-cell trait contain an abnormal haemoglobin (haemoglobin S). When these cells are incubated under conditions of reduced oxygen tension, such as by sealing a wet film between two slides, sickling occurs, the process taking from 15 minutes to several hours. The cells may show irreversible sickling, but usually revert to their normal shape on the admission of air. If, therefore, the slides are separated to prepare a blood film, which is fixed and stained in the usual way, the cells will be found to present a normal appearance. Attempts to fix the cells before separating the slides is likewise unsuccessful, since no cells remain after staining.

In view of the comparative rarity of the condition in the United Kingdom, a stained preparation would be useful for demonstration purposes, and it has been found possible to do this by the following method.

Method

A drop of oxalated blood was placed on a slide and covered with a second slide. The cells were observed under the microscope until sickling occurred. Two tablets of paraformaldehyde on a piece of wire gauze were placed on a tripod and heated with a Bunsen burner until a concentrated stream of formaldehyde was being evolved. The two slides were then rapidly separated to produce blood films and held face downwards in the formaldehyde. Fixation was immediate and drying was rapid. They were then stained with Leishman's stain.

I wish to thank Mr. F. W. Eels for his assistance in finding this method.

The next number of the *Journal* will contain the papers read at a Symposium on the Pathology of the Cell organized by the Association of Clinical Pathologists in honour of the centenary of the publication of Virchow's "Cellular Pathology."

J. clin. Path. (1958), **11**, 458.

BOOK REVIEWS

Laboratory Applications in Clinical Pediatrics. By Irving J. Wolman. (Pp. xi+1019. £5 16s. 6d.) London: McGraw-Hill. 1957.

In his introduction the author explains that this book has been written to enable the paediatrician to become familiar with the scientific meaning and clinical applicability of the many test procedures. Thus he should have some understanding of the biochemical, cytological, and microbiological processes being assayed by laboratory analyses. The book is not intended for the practising laboratory worker, but for the general practitioners and students who are dealing with children. The mode in which the problem is tackled varies somewhat. At times, separate investigations such as haemoglobin level are discussed; at others, distinct disease conditions. To some extent it is unavoidable in a book of this character that there should be this double method of approach, and Professor Wolman has been more successful than many in avoiding repetition and at the same time keeping together the investigations of a given disease.

The book starts with 27 short chapters on haematology. Much of this is excellent, but the author has not solved the problem of how much technical description to put in. There are quite lengthy passages dealing with techniques which would normally be beyond the performance of the people for whom the book is intended, yet insufficient for the laboratory worker. In dealing with abnormal cells, particularly in the bone marrow, there are long passages describing these cells, but no illustrations. One feels that much of the technical description in these chapters could have been shortened. However, the information on normals at different ages and the significance of variations found are admirably dealt with.

The next seven chapters deal with urine and urine analysis. Again there is this slightly uneven balance, for example, the Sulkowitch test is described in detail and then dismissed as "not very reliable as a screening test." It is difficult to see why the author in dealing with glycosuria interpolates a short essay on the management of diabetes. Some of the normal values differ from the accepted, for example, for boys an average creatinine coefficient of 25 seems to be unduly high.

The chapters on kidney function are adequate, but the rapid survey of the various conditions in which disturbances of the tubule functions occur have but little relationship to laboratory application, and one feels a clearer-cut directive as to how one is to identify the various conditions would be of greater value than the series of thumb-nail sketches which are actually given. There are five chapters on cerebrospinal fluid. Indications for examination and methods of obtaining specimens of cerebrospinal fluid from different sources are clearly given. A rapid method for estimation of glucose is described, but the actual method of reading the result is not very clear. The various types of infection are discussed and this section is quite helpful.

The next four chapters are on the liver and its diseases. One must admit that the application of the laboratory methods to the diagnosis of liver diseases, especially in the first year of life, is difficult and unrewarding, but for a book of this kind one feels that this problem has been rather superficially dealt with.

The next 12 chapters deal with blood chemistry. The chapter on blood glucose is excellent and the various techniques are well described. The chapters on lipids, protein, nitrogen, and plasma proteins are straightforward and the necessary information is given without elaboration. There is a useful chapter on blood iron. The chapter on calcium also covers the ground, but is somewhat uneven. Potassium is given a chapter on its own, but the treatment is somewhat stereotyped and the statement that in the absence of a flame photometer the electrocardiogram is the quickest process for detecting an abnormal potassium level is open to question.

Four chapters on body water and electrolytes follow in which the problem is dealt with in a simple but adequate manner, a reasonable balance being struck between the slide rule and the rule of thumb in the management and correction of electrolyte disturbances in infants.

The next chapter on vitamins and their blood levels is rather superficial. There are five chapters on the gastro-intestinal tract, and again this is adequately covered.

The endocrine system is dealt with in five chapters; thyroid, adrenal cortex, ovaries and vagina, testes, and pituitary. This section definitely falls below the level of the previous ones, and the reader will gain only limited help from it. This is particularly unfortunate because an adequate description of the specific tests and methods of investigation in childhood is needed, and one feels that here, largely, the information has been obtained from adult medicine and not especially adapted to paediatrics. For example, only a brief reference is made to the adrenogenital syndrome and no adequate advice is given as to its early detection.

The next seven chapters of approximately 150 pages are given to infectious diseases, and a discussion of the types of bacteria, virus, and organisms which can infect the body. One feels that in these chapters the difficulty of deciding whether one is talking to a clinician or to a class in elementary bacteriology becomes manifest, and the impression is gained that the author

had no clear idea which group he was dealing with, with the result that there are many pages in which a purely bacteriological aspect is taken without being adequately balanced by a clinical appraisal.

There are three chapters on chemotherapy which illustrate the somewhat different approach of our transatlantic colleagues to the problem, but on the whole the information and advice given are very sound. Chapter 29 on obscure generalized diseases seems to be too brief to be of any particular value, and likewise the chapter on poisoning is too short and superficial to be of great use.

There are many misprints in this book, some of which are more than a nuisance inasmuch as it is not obvious exactly what is meant. For example, on p. 523 the ratio of ester to total cholesterol should be ester to free cholesterol. Another irritating habit of the printer is to break a word up at the end of the line in an unusual manner—for example, methemalbumin is recorded as methe-malbumin. On the credit side there are many references at the end of each chapter. These show a welcome spread into other areas besides America, and other continents are well represented.

It is rather difficult to give an overall impression of this book. Some parts are excellent. An author of the reputation and experience of Professor Wolman could hardly write a book of this length without including much of value for any reader. However, unless the American clinicians have more time for reading long books than their British counterparts, it is difficult to see how a clinician can gain enough from this book to warrant reading it from cover to cover. If used as a reference book for help in specific problems it is inadequate in too many parts for this purpose, but it will give the needed references. The clinical pathologist can undoubtedly learn much from it which would be helpful in dealing with his younger clients, but he will often find he has to consult some other source for the actual information he needs.

W. W. PAYNE.

Connective Tissue: A Symposium Organized by the Council for International Organizations of Medical Sciences. Established under the joint auspices of UNESCO and WHO. Edited under the direction of R. E. Tonbridge. (Pp. xii+371; illustrated. 42s.) Oxford: Blackwell Scientific Publications. 1957.

This comprehensive symposium was held in London in 1956, and the book summarizes the carefully evaluated work of some 30 participants on the biochemistry of connective tissue, with particular reference to the correlations between biochemistry and structure. The subjects cover a wide field and clearly illustrate the progress that has followed the application of *x*-ray diffraction analysis, new biochemical techniques, and electronmicroscopy, with its inherent difficulties.

The monograph will be of great value to the pathologist who has been studying the biology of connective tissue or is interested in the technique and methods applied. He will be fascinated by the complexity of the connective tissue system and its three main components, the cells, ground substances, and fibres. The correlation between biochemistry and structure, with the help of the electronmicroscope, has made some progress, but there remain many problems to be solved. The main difficulty, as emphasized by several authors in this symposium, lies in the fact that the procedures of extraction might greatly affect the final result. Moreover, as in other fields, definitions and terminological difficulties may hamper understanding. This is reflected both in the masterly introduction of W. T. Astbury and in the Chairman's summing-up by R. E. Tunbridge. To quote from the latter, "the complexities of the structure, composition, and function of collagens and allied fibrils, remain all too apparent, but a much greater understanding of one another's difficulties and objectives has resulted from this symposium."

I. FRIEDMANN.

Microtechniques of Clinical Chemistry for the Routine Laboratory. By Samuel Natelson. (Pp. xv+484; 160 figures. 82s. 6d.) Oxford: Blackwell Scientific Publications; Springfield, Illinois: Charles C. Thomas. 1957.

Dr. Natelson is well known in the United States for the leading part he has played in the development of small-sample analysis in clinical chemistry. This manual contains methods for determining some 70 constituents of blood or urine, requiring in most cases no more than 0.01–0.1 ml. of material. There are sections on general principles of technique in this field, and on apparatus such as micropipettes, microburettes, and balances used for measuring with precision the small quantities involved. Nearly all these are of American manufacture. An appendix contains, among other things, a critical evaluation of laboratory error and an account of absorption spectrophotometry.

The methods have been well tried in the author's laboratory and have proved reliable and easy to perform. On the whole they are up to date, but a few of the older techniques, e.g., icterus index and the Folin and Wu blood sugar method, would have been better omitted. It is a surprise to find a serological test for syphilis included. Helpful comments on procedure are given, but the principles of the methods are not always stated clearly, and the normal range is sometimes omitted. It is apparent that the book is intended to be used in conjunction with and to supplement standard technical manuals, for little is said about precautions in collection of specimens, function tests, etc. Sometimes alternative methods are described in detail, and as many as 20 to 30 references to other methods are given for each constituent, making this a good bibliographical source of chemical pathology technique, micro or otherwise.

Few books at the present time deal specifically with the problem of quantitative micro- and ultramicro-analysis for hospital laboratories and this is a welcome arrival in this field. It can be recommended especially to the attention of pathologists and biochemists connected with paediatric units.

W. H. R. AULD.

The Chemistry of Blood Coagulation. By Paul Morawitz; translated by Robert C. Hartmann and Paul F. Guenther. (Pp. x+194. 34s.) Oxford: Blackwell Scientific Publications; Springfield, Illinois: Charles C. Thomas. 1958.

The translation of this classical monograph is the most recent of the American Lecture Series and is a distinguished addition. The monograph is of much more than merely historical interest, for a good deal that is "new" in this field to-day is clearly the expansion, development, or modification of hypotheses initially explored by the great investigators of the past. This monograph presents an absorbing review of their work in the field of blood coagulation in the late nineteenth and early part of this century. The authors have, therefore, performed a useful practical service to those engaged in haematology to-day. Many unlikely to read the original text in German will find in the translation a welcome ease of assimilation, for the authors have achieved an imperceptible translation of a truly remarkable review of a very complex subject; remarkable in that the subsequent great developments in this field should so enhance the reputation of the original author. A publication of this kind has a general educative value and the publishers hope that the translation will appeal, among others, to the medical student; but the pressures in modern medicine which restrict the student's general education in other directions are likely to operate here also, and educated physicians become less numerous as time goes on. It is to be hoped that this translation will meet with the acceptance it has earned, and that it will be read by at least those who practise haematology. There is an extensive bibliography.

H. E. HUTCHISON.

'Analar' Standards for Laboratory Chemicals, 5th ed. revised. (Pp. xvi+397. 21s.) Formulated and issued jointly by the British Drug Houses Ltd. and Hopkin and Williams Ltd. London: Eyre and Spottiswoode. 1957.

The two firms which independently produce and market "Analar" chemicals first published this handbook on their products in 1934. In it they defined their standards of purity for each compound, and described in detail how the purity of these chemicals could be checked. In this new edition a number of reagents appear for the first time, the text has been generally reviewed and improved, and in general the preparation of the book is in keeping with the good reputation enjoyed by the firms. One notes, however, that with some of their compounds only a small proportion of the total impurities present are given, e.g., sodium bismuthate is guaranteed 83% pure, yet the only impurities listed (chloride and manganese) total 0.01%.

The book serves as a useful record of the main properties of the substances described and could be used for reference in qualitative and quantitative analysis. Some of the tests are original and all have apparently been the subject of extended experiment in the analytical laboratories of the manufacturers. The appendix contains notes on the preparation of a large number of test reagents, indicator solutions, standard solutions, etc.

W. H. R. AULD.

Transactions of the American Goiter Association 1956. (Pp. xiii + 473; illustrated. £6 6s.) Oxford: Blackwell Scientific Publications; Springfield, Illinois: Charles C. Thomas. 1957.

This book contains the 38 papers given to the 1956 meeting of the American Goiter Society with the discussions to each. All except seven of these have been published either in *The Journal of Clinical Endocrinology and Metabolism* or in *Endocrinology*. Naturally the rapid increase in knowledge of the thyroid hormones and thyroiditis would call for some changes now, but there are many points of all-round interest.

Thyroid preparations from swine are commonly twice as potent as from cattle. Goitrous sheep used to be common but have almost disappeared as a result of feeding iodized salt.

Cobalt exerts a direct effect on the thyroid cell characterized by simultaneous inhibition of endocrine function and increased phosphorus uptake. A narrow range of cobalt dosage may be a critical factor in goitrogenesis.

Patients with hyperthyroidism are hardly ever alcoholics. Rats made hyperthyroid have a decreased appetite for alcohol, an increased appetite for stock diet and sugars, and an increased thirst for plain water.

PBI^{131} studies suggest that children up to 4 years of age are in a state of thyroid "hyperactivity" when compared with euthyroid adults.

An iodinated substance, compound X, has been found in the serum of some patients with cancer of the thyroid; it is similar in all cases studied.

In the diagnosis of hyperparathyroidism demonstration of subnormal tubular reabsorption of phosphate (T.R.P.) is of value. On a low-phosphate diet the serum calcium level is increased to a greater extent and T.R.P. to a lesser extent than in normal individuals.

Tri- and tetra-iodothyroacetic acid give an immediate response in cases of myxoedema,; it ceases within 24 hours. Thyroxine elicits little or no response within 24 hours. This suggests that the acetic acid analogues are the form in which thyroid hormone acts in tissues.

Cretinism has many causes. Simplest is congenital absence of the thyroid. Symptoms are not as severe in patients with aberrantly located fragments of

thyroid tissue. Endemic cretinism is associated with severe endemic goitre. Metabolic cretinism is due to defective synthesis of the thyroid hormones; failure of iodide oxidation in the gland, failure of coupling of hormone precursors into hormones, escape and loss of iodotyrosines because of failure of normal deiodinating processes.

Low thyroid reserve most frequently follows treatment with I^{131} or subtotal thyroidectomy. It has also been seen after thyroiditis, and shortly after cessation of prolonged thyroid medication. It may be transient or permanent.

In 25 years the Mayo Clinic saw 30 small-cell malignant lesions; these consisted of 18 malignant lymphomas and 12 small-cell carcinomas. The average age of the patient in the former case was 56 and in the latter 53.5 years. Small-cell malignancies are uncommon before 40 years. Prognosis is much better for the lymphomas; six of the 18 patients were alive and well four years or more after they were first treated. No patient with small-cell carcinoma survived more than 19 months. Surgical excision of malignant lymphoma should be vigorous even in the presence of invasion of muscle and tissues around the trachea and oesophagus; it should be followed by radiotherapy.

Repeated stimulation of thyroid cancer to induce greater uptake of radioactive iodine is most unwise, as it might enhance autonomy of growth.

Riedel's struma is a very rare disease. Its chief characteristic is the invasive fibrosis that surrounds and obliterates the thyroid. Only eight cases have been seen in 34 years at Presbyterian Hospital, and 20 cases in 36 years at the Mayo Clinic.

The paper on nodular lesions of the thyroid in children from the Mayo Clinic is most valuable. Before 1935 many children seen had large colloid goitres with poorly defined nodules; these sometimes responded to treatment with desiccated thyroid or iodine. Since 1935 these cases have disappeared and carcinoma is the commonest cause of nodular enlargement. Hence the preferred treatment is surgical. Lymphocytic thyroiditis was seen in eight children between 8 and 14 years. The gland was two to three times normal size and felt nodular from an exaggeration of the normal thyroid lobulation. Goitrous cretinism and congenital goitre are other benign causes. In 47 years 46 cases of cancer have been seen in children below the age of 14. In every instance the presenting symptom was goitre, or, in no less than 36 of the 46 cases, enlargement of one or more cervical lymph nodes from secondary deposits. All except three of the 36 with metastases had palpable abnormalities of the thyroid. The common form of cancer (41 out of 46) is a slow-growing papillary and follicular adenocarcinoma which metastasizes early to lymph nodes and much later to lungs and occasionally to bones and other structures. The other form is adenoma malignum. The prognosis after surgical treatment is relatively good. Many of the patients with cancer gave histories of having had irradiation before the disease was discovered; in one child thyroid cancer developed 15 years after irradiation of a lymphangioma of the tongue. Both carcinoma of the thyroid and thyroiditis have been seen with much greater frequency during recent years. From 1908 to 1919 cancer accounted for 4.5% of the cases of nodular goitre in children, but from 1950 to 1955 for 70%.

Below the age of 40 most cancers of the thyroid are similar to those in children and have a favourable prognosis with early treatment. In the 40–60 age group thyroid cancer is a more important cause of death as they are more invasive. After the age of 60 unsuspected cancer becomes rare but cancer itself of great importance. Most of these are indifferentiated and very malignant.

G. R. OSBORN.

Clinical Biochemical Methods. By A. Tarnoky. (Pp. viii+239; 11 figures. 50s.) London: Hilger and Watts. 1958.

New books on laboratory techniques must be judged largely on their superiority over existing books in scope, selection of methods, and adequacy of description. This book sets out only to describe the biochemical analyses in routine use in the laboratory of the Royal Berkshire Hospital, and therefore the choice of methods, although fairly wide, has rather a local bias. Thus methods are given for estimating urinary dehydroepiandrosterone and "urine pigment" and for a "four-point glucose test," but not for blood pyruvate or barbiturate or for serum iron. A surprising omission is the chromatography of urine sugars, especially as no mention is made of the specific enzyme test papers for glucose. On the other hand, the formol gel test and tests for urinary indican could probably be omitted from a book which does not attempt to be comprehensive.

The methods selected are mostly sound and well established. They are restricted to those using apparatus which is not "expensive or highly specialized." Thus no spectrophotometric methods are included, but oddly the estimation of CO_2-combining capacity is given by the Van Slyke manometric rather than the much commoner volumetric apparatus. Photometric methods are described only in terms of the Hilger "biochem" absorptiometer, and those who do not possess one may be uncertain which of their own filters corresponds with "filter No. 55." It is perhaps doubtful whether a colorimetric method of *p*H for venous blood using a Lovibond disc, with a normal range of 7.3 to 7.5, can be of very much clinical value. The estimation of trypsin by serial dilution and gelatin liquefaction could well be replaced by one of the more quantitative methods. The method given for distinguishing methaemoglobin from sulphaemoglobin recommends using a reducing agent, which can give false results and is better replaced by cyanide solution.

The layout is clear, with generous spacing which leaves ample room for marginal notes. Each method starts at the top of a page, and most methods can

be seen in full without turning a page. The description of each method is given step by step, but rather concisely. Formulae for calculating results are given without explaining their derivation. In none of the colorimetric methods is any mention made of the range over which Beer's law is obeyed, and in most only a single standard is recommended, of a value well below the upper range found in patients. It is a surprise to find that under the Nesslerization techniques for blood urea and plasma proteins only ammonia standards are mentioned; however, tucked away inconspicuously at the end of the book one finds a most important section on analytical control which gives some useful hints on the maintenance of accuracy, and here the importance of standard protein and urea solutions is acknowledged. A good point is the information on the accuracy to which reports should be given, e.g., faecal fats to the nearest 1%. Only one misprint was noted, that maximal urea clearance is "proportional to" instead of "independent of" urine minute volume.

This, then, is another practical bench book which gives concise descriptions of most of the biochemical investigations carried out in the average hospital laboratory, but which has rather a local flavour. Like other books of this type, it devotes little space to errors which may arise and methods of preventing them. Thus the reader derives little benefit from the experience of the author, and will have to learn the hard way that, for instance, the specific gravity method for plasma proteins gives high results in uraemia, or that traces of acetone can ruin a Nesslerization method, or that errors in urine specific gravity will result if the temperature of the urine is different from that at which the urinometer has been calibrated. It is arguable that such books no longer meet the needs of present-day laboratories. At a time when the errors which may arise in routine chemical analysis are becoming more widely recognized, laboratories are faced with a steady increase in the range and complexity of biochemical investigations and a shortage of skilled and experienced technicians. This calls for a much fuller description of methods, which should incorporate the practical experience of the best laboratories, with full details of possible errors and their prevention. Something on these lines has already been achieved in *Standard Methods of Clinical Chemistry*, and it is to be hoped that this example will be more widely followed.

G. K. McGowan.

Association of Clinical Pathologists Broadsheets

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A SYMPOSIUM IN HONOUR OF THE CENTENARY OF VIRCHOW'S "CELLULAR PATHOLOGY" (1858-1958)

At the instigation of the Council of the Association of Clinical Pathologists the Editorial Board decided to devote this issue of the Journal to the publication of this Symposium on the pathology of the cell, which was held at the Royal College of Surgeons on October 2, 1958, to commemorate the centenary of the publication of Virchow's classical volume. Participants of different interests in the cell were invited to contribute, and consequently it is hoped that in this issue readers will receive a wide and stimulating survey of the developments and the newer knowledge on the fundamental aspects of the physiology and pathology of the cell now being pursued.

1858

BY

SIR ROY CAMERON, F.R.S.

From University College Hospital, Medical School, London

"For my part I am ready for anything, at a time when asphalt, India rubber, railroads, and steam are changing the ground we walk on, the style of overcoats and distances." So remarks one of Balzac's characters in his *Deputy from Arcis* as he curtly dismisses the 1830s. It was a time of transition from the splendours and miseries of the Napoleonic era to the calm of the later Victorian period, ruffled only by local revolution and minor wars. Technical invention had infiltrated art as well as ordinary existence. The invention of photography led to the dissemination of copies of great paintings and the revelation of styles. And when the American painter Rand found out how to make a collapsible tin tube whereby paints could be stored indefinitely and ready for immediate use, the mode of painting changed and Romanticism and Impressionism were born. This, too, was the age of the supreme romanticists in music, Robert Schumann, Johannes Brahms, and above all, Hector Berlioz and Richard Wagner. The Impressionists rejected the anecdotal and planned picture. "The great scandal of their painting was not that their pictures were bright," writes Grosser, "but that they were unpremeditated." Music broke from classical form and, with poetry, dared to give voice to the innermost emotions and flights of mind.

With the Congress of Vienna in 1815 was ushered in, for western Europe, 99 years of freedom from long and devastating wars. Populations expanded as wealth and comfort increased in some communities; in others, condemned to ignorance, poverty, and despair, they were fostered by better medical knowledge, and the breakdown of old customs and restrictions, early marriage, and the like.

Nowhere was this change more apparent than in Germany. In the west, the traveller encountered a framework of rural life where compact villages were grouped around open fields or hamlets and scattered farmsteads stood in marshy valleys. As he journeyed eastwards, the more apparent it became that he was in a region conquered from Slavs. Most of the land was in the hands of the lord of the manor, vulgarly called the junker, who rigidly insisted upon a wide distinction between his family and the peasants. Many of the latter were no better than serfs, amenable to manorial jurisdiction and bound to the soil. In some of the quaint little grand duchies, electorates, and free towns of old Germany he was not too heavily taxed and life was tolerable though liable to sudden upsets according to the whim of the ruler. Many of the towns were, indeed, quiet backwaters of huddled roofs and spires, and even in 1850 the 12 largest towns of Germany accounted for a mere million and a third of people. Paris alone at this time had more than a million citizens.

With the effective exploitation of the Ruhr coalfields, the development of railroads and steamboats, the institution of a Customs Union in 1834, and the standardization of the coinage came a subtle change. The first German railway was opened in Bavaria in 1835 despite the violent opposition of the Bavarian College of Physicians; in 1839 the newly completed line from Leipzig to Dresden carried more than 400,000 passengers in its first year, including ladies who kept needles between their lips to check familiarity in the single tunnel. By the 1850s, 3,000 miles of railroad had been built at an average cost of less than £11,000 per mile as compared with £40,000 in England. This meant more food, and with the introduction of chemical manures, as the result of Liebig's teaching in 1840, most of the sandy or heath-covered north German plain was at last accessible to cultivation.

Almost inevitably with these changes came labour troubles and the storms of revolution. The antiquated, absolutist bureaucracy encountered growing opposition from Liberals and Marxists. Unrest in the smaller states and the influx of political fugitives from France and Switzerland shook time-honoured dynastic loyalties, and the revolution that broke out in Poland in 1846, and swept through Paris in the February of 1848, flared up in Berlin in March of that year. Barricades appeared in the streets overnight and fighting broke out between the angry proletariat and the Prussian dragoons. King Frederick William IV was compelled to give way before the storm, a liberal, reforming ministry was appointed, and the King retired to Potsdam. In 1857 he went mad and his brother William took over the Regency. A rigid conservative, William was fully convinced of the divine rights of kings, but he detested police government. In 1858 he agreed to some reforms and seemed inclined towards a more liberal policy, but, reactionary elements prevailing, and ignoring the advice of his friend the Prince Consort of England, he reverted to the old policy of military alliances with German princes, absolute monarchial powers, and Army control. The same reactionary spirit prevailed in public health affairs, and Rudolf Virchow, in his capacity of Berlin politician and eminent scientist, played a foremost part in the fight for reform, just as he had rallied to the barricades with other ardent young Berliners in the turbulent weeks of 1848.

1858

No great perturbation shook the earth, no comet blazed its portents in the sky in that leisurely year of 1858. The British had captured Lucknow and rolled up the map in India for a few uncertain years when Lord Canning, the first Viceroy, declared an amnesty for the mutineers. Orsini tried to assassinate Napoleon III. The Princess Royal, Queen Elizabeth's eldest daughter, married Prince Frederich Albert of Prussia. China surrendered the left bank of the Amur River to Russia, and Jews were allowed to sit in the English Parliament. Yellow fever broke out in Lisbon in 1857 and so panicked the city that 50,000 inhabitants emigrated in a hurry. The Reverend Dr. Livingstone set out for Africa again, and a Jewish gentleman named Bravo brought an action against the Eastern Counties Railway Company for injuries sustained when the carriage into which he was stepping was suddenly propelled forwards. He was prevented for some weeks from performing the rite of circumcision, an operation which the *Lancet* characterized as one requiring "considerable nicety and skill." Professor Faraday declined the offer of the Chair of Chemistry at Edinburgh, which action the *Lancet* approved since "in these days the glories of the modern Athens have somewhat paled." A hippopotamus in the Paris menagerie gave birth to a calf which began immediately to swim about in the water in which it was born, "his voice being so strong an hour after his birth that people mistook it for that of the parents." Sir Benjamin Brodie, a surgeon of the highest eminence, was elected President of the Royal Society, and Mr. James Nolan of Carlow, Ireland, died at the age of 115 years and 9 months. Scotland achieved an unenviable pre-eminence in drunkenness, according to lunatic asylum and police returns.

> "Even in Murray's Asylum, where there are so many patients of the higher classes that Dr. Lauder Lindsay says 'this circumstance gives a pleasing tone to our society,' the percentage of cases arising from intemperance is nearly double that of the Surrey County Asylum, which is wholly devoted to paupers. . . . For every person taken into custody in London for being drunk and disorderly, two are taken into custody at Edinburgh and three at Glasgow."

Captain Harrington of the good ship *Castilian*, hard by St. Helena, saw the sea serpent "lying like some large Doric column upon the waters, stretching away 200 feet or so in length, and 10 in diameter. He hastened home, and through the universal *Times* imported the intelligence to the wide world." A letter appeared in the selfsame *Times*, pleading for "a fig-leaf" to cover the regimental recruits when undergoing medical inspection preliminary to enrolment. The sanitation of many great cities was being heatedly debated. London was no exception, and Dr.

Barker of Bedford described experiments in which he exposed rats, mice, and dogs to the effluvia of sewage gas which "grew sick, restless, and diarrhoeal"; the *Lancet* sourly comments:

> "It is no other than happens to the 2 millions of human beings who in London live in close chambers over sewers and cesspools. . . . The hideous—the truly horrible, state of the river Thames" was at last "exerting a feeling of public indignation which will not be easily repressed." Someone called Kahn opened an exhibition in Coventry Street containing "additions of a most disgusting character . . . and days were set apart for the admission of 'ladies.'"

The abominations of the Strand slaughterhouses were arousing public indignation.

> "The poisonous fumes from open cesspools pollute the air of the cellars in which the beasts are kept during two or three days, and in which, after being slaughtered, their flesh is suspended. The evil does not end here, for the putrefying offal is thrown into the sewers of this crowded neighbourhood, and become a fresh centre of infection."

Yet, according to the *Lancet:*

> "the winter has been so unusually mild that the health of the people stands more than commonly high, while half the crowned heads of Europe have been on the sick list. The King of Prussia is suffering from a severe cerebral affection, from which no favourable issue can hardly be anticipated. The Grand Duke Ludwig of Baden, who has long been under strict medical surveillance,

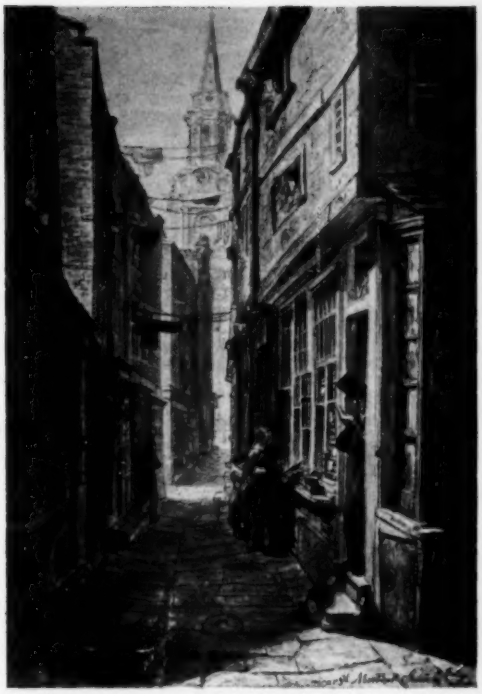

FIG. 1.—London: St. Martin-in-the-Fields in 1828 (painting by G. Scharf).

FIG. 2.—London: the Strand from the corner of Villiers Street (painting by G. Scharf).

FIG. 3.—London: old Covent Garden market in 1825 (painting by G. Scharf).

FIG. 4.—London: a dairy in 1825 (painting by G. Scharf).

died last week. The King of Sweden, slowly recovering from a severe illness, was thrown back by an attack of fever early last month, from which he is more convalescent. The King of Wurtemburg is reported to be fast breaking; he recently suffered from an attack of 'grippe.' Such a list affords ample food for the moralist."

Robert Brown, the eminent botanist and explorer, whose name has achieved immortality in "Brownian movement," Johannes Müller, renowned for his researches in zoology and embryology, Robert Hare, the inventor of an oxyhydrogen blowpipe with which he was able to fuse platinum, and Baron von Welsbach, who invented the incandescent gas mantel, died in this year. Births included those of Max Planck, whose law of radiation brought about a major revolution in scientific thought, Christiaan Eijkmann, the co-discoverer with Gowland Hopkins of vitamins, E. M. Crookshank, who founded in King's College, London, the first bacteriological laboratory to be established in Britain, Rudolf Diesel, who built the first successful diesel engine in 1897, Robert Hadfield, the discoverer of manganese steel, and Leoncavallo and Puccini, the Italian composers. In this selfsame year, too, Charles Dickens was writing *A Tale of Two Cities*, Anthony Trollope completed *Doctor Thorne*, and George Eliot was at work on her *Mill on the Floss*. Richard Wagner had just written *Tristan and Isolde*, Samuel Clemens was piloting a Mississippi river steamer and steadily moving forward to his literary rebirth as Mark Twain. On July 10 Charles Darwin and Alfred Russel Wallace communicated their papers on evolution and natural selection to the Linnean Society of London, and Rudolf Virchow's "Cellular Pathology" was published in Berlin on August 20.

Of London itself, much has been written. An observant, fastidious young American, Henry Adams, who visited the city in 1858, found in

> "the long, muddy, dirty, sordid, gas-lit dreariness of Oxford Street . . . a certain style (that) dignified its grime. . . . The eighteenth century held its own. . . . Vanity Fair was alive on Piccadilly in yellow chariots with coachmen in wigs, on hammer-cloths, footmen with canes, on the footboard, and a shrivelled old woman inside; half the great houses, black with London smoke, bore large funereal hatchments, every one seemed insolent, and the most insolent structures in the world were the Royal Exchange and the Bank of England." (Figs. 1–4.)

An amalgam of well-nigh unlimited wealth and grinding poverty, of luxury and Victorian stability

FIG. 5.—Berlin: Unter den Linden in 1829 (aquarelle by E. Gaertner).

FIG. 6.—Berlin: view towards the Royal Palace from Unter den Linden in 1853 (oil painting by E. Gaertner).

FIG. 7.—Berlin: Parochialstrasse in 1831 (oil painting by E. Gaertner).

side by side with filthy stews, misery, and starvation, it was no better nor no worse than a dozen cities of its rank in Europe and America.

Berlin in 1858

Built on a barren, sandy plain without a vestige of a hill and traversed by the muddy, sluggish Spree, Berlin is oppressively hot even in May and bitterly cold during the long-drawn-out winter. To young Henry Adams of Boston it appeared as

> "a poor, keen-witted, provincial town, simple, dirty, uncivilized, and in most respects disgusting. Life was primitive beyond what an American boy could have imagined. Overridden by military methods and bureaucratic pettiness, Prussia was only beginning to free her hands from internal bonds. . . . German manners, even at Court, were sometimes brutal, and German thoroughness at school was apt to be routine. . . . The condition of Germany was a scandal and nuisance to every earnest German, all of whose energies were turned to reforming it from top to bottom." Adams came to appreciate the "simple character, the good-natured sentiment; the musical and metaphysical abstraction; the blundering incapacity of the German for practical affairs. . . . Germany had no confidence in herself, and no reason to feel it. She had no unity, and no reason to want it. She never had unity. . . . Until coal-power and railways were created, she was mediaeval by nature and geography."

But a certain simple charm emanated from the well-planned streets of Berlin, lined as they often were with lime trees beneath which the people sat

FIG. 8.—Berlin: Wilhelmstrasse and Wilhelmplatz in 1855 (steel engraving by Borchel).

FIG. 9.—Berlin: street corner near Royal Palace in 1830 (oil painting by J. E. Hummel).

at night listening to the band. Entrance from the west by the Brandenburg Gate, an imitation of the Propylaeum of Athens, with its large figure of Victory in her chariot drawn by four horses and bearing the Prussian eagle, led to the broad Unter den Linden and the royal palace (Figs. 5 and 6). Many of the buildings were imposing and built of brick and stucco. The old or eastern part of the city was given up to business, and was traversed by long, stone alleys, with footpaths on either side raised above the centre or separated by a kennel lined by small rough stones (Fig. 7). There were many squares (Figs. 8 and 9), some spacious and surrounded by tall, handsome buildings with little or no ornaments, seldom paved or turfed and often treacherous from stagnant water. The left bank of the Spree was dead flat so that its water often overflowed the pavements.

Between the Brandenburg Gate and the palace were crowded together the guard house, the university, arsenal, opera house, and the new theatres. The palace was a roomy plain building, fronted with large parade grounds. Outside of the Brandenburg Gate stretched an extensive plantation bounded to the south by small handsome villas and on the north by the Spree, whose bank was dotted with coffee houses, rustic benches and tables beneath limes and elms, and numerous beer houses. This was the famous Thiergarten much frequented by squads of schoolboys and beloved by the Berliner and his family, rivalling in his favour the Palace of Charlottenburg, two miles from the city, where Frederick the Great had kept his sculpture. A small Doric temple, among cypresses and weeping willows, marked the tomb of Queen Louise of Prussia, who had rallied the people against Napoleon.

In spite of the general air of cleanliness and order, Berlin had its black spots. Even in 1873, as young William Osler wrote:

> "The drainage is everywhere deficient, and in the greater part of the city the sewers are not even covered but skirt the pavement on each side, sending up a constant odour, which until one gets acclimatized is peculiarly disgusting."

The incidence of typhoid fever and kindred diseases was high and epidemics were frequent. Poverty and crime were common enough, and concentrated in those areas where the railways crept inwards from the four points of the compass. The journey from London to Berlin, via Ostend, a distance of 765 miles, took 40¼ hours. Hospitals and charitable institutions were numerous, but medical teaching was centralized around the Royal Charité. A sequence of illustrious teachers had, however, made Berlin the focus of the medical scientific world. The newly erected Pathological Institute at the Charité, presided over by Virchow, was a Mecca for pathologists. A heavy drinking, smoking, duelling set of students thronged the lecture rooms and wards. To young Osler they seemed

> " a hard-working set, much given to long hair and slouched hats, and a remarkable number of them wear glasses. They possess the virtue, quite unknown as far as my experience agrees, among their English or Canadian brethren, of remaining quiet while waiting for a lecture, or in the operating theatre. . . . Field sports, such as cricket and football, are entirely unknown among the students, but they have a curious habit of forming small societies of 10 or 12, who have a room at some restaurant where they meet to drink beer, smoke, or discuss various topics. . . . Drunkenness is not common, at least not obtrusively so, but they appear to get a fair number of cases of delirium tremens in the Charité."

For those who lived in a residential institution such as the training school for army surgeons, where Virchow received his education, life was severely disciplined. The lecture time-table is positively frightening. The youthful Virchow and his fellow student Helmholtz slogged away at 48 lectures each week with 12 hours of revision classes. Helmholtz wrote to his parents in 1838, " The food in the Institute is not so bad as most people make out,* though less good than in a private home. We can have two helpings of soup and vegetables, but only one of meat. Or instead of vegetables we may have sauce over the meat, with potatoes." Helmholtz shared a large room with one or two others, had his own piano on which he played the sonatas of Mozart and Beethoven, and ran a kind of glee club. In the evenings he read Goethe and Byron and sometimes integral calculus ! Apparently he was not regarded as a prig or freak by the tough army set. The numerous letters of young Rudolf Virchow to his parents, though harassed by the educational treadmill, give no hint of unhappiness or persecution.

*Largely sauerkraut, sausage, and beer, so Henry Adams assures us. A far more serious feature was the dislike of fresh air.

Virchow in 1858

This selfsame Rudolf Virchow was born in 1821 in the tiny Pomeranian county town of Schavelbein. His father was a small farmer who dabbled unsuccessfully in petty commerce. Of Virchow's mother we know scarcely anything. She seems to have been a fretful, worrying woman who played little part in the development of her son. Their life was narrow and colourless and copied that of the junkers of eastern Germany, from whom was emerging that man of iron will, Bismarck. Contemporaries of Virchow included Helmholtz, the physicist, Schliemann, the excavator of Troy, Flaubert and Baudelaire, the French writers of genius, and Mary Baker Eddy, the founder of Christian Science. Virchow was slightly junior to Claude Bernard, Karl Marx, Queen Victoria, and John Ruskin, and slightly older than Louis Pasteur, Francis Galton, and Lord Kelvin.

A school career at Cöslin marked Virchow as a brilliant scholar and a rebel against authority. Despite strong competition, he gained a place at the Friedrich-Wilhelm Institute in Berlin in 1839, where he underwent training as an army doctor. From the Institute came Helmholtz, Loeffler, Gaertner, Behring, and many distinguished doctors, yet instruction was largely empirical and speculative. But though training was stern and harshly Prussian, with long hours of study and little leisure, there was some let-up from time to time, as Helmholtz's account of his life shows, and the dogmatic classes and revisions were lightened by the university lectures of three of Germany's greatest medical teachers, Johannes Müller, the physiologist, J. L. Schoenlein, the clinician, and Casper, the toxicologist. To the former, young Virchow owed his initiation into scientific method ; a letter to his father exists in which he asks that some rabbits be secured for some experiments he wished to conduct when he came home.

Qualifying in 1843 at the age of 22, Virchow served as " company surgeon " in the Charité Hospital, where he picked up some chemistry and histology from Robert Froriep, the prosector, whom he succeeded three years later. Meanwhile, his reputation as a teacher mounted, and in 1845 appeared his first publication on fibrin and " white blood," forerunner of his justly celebrated work on embolism and leukaemia. On May 3, 1845, in an oration commemorating the birthday of the Friedrich-Wilhelm Institute he urged that all sorts of methods be employed in extending clinical research, stressing the impor-

tance of physical and chemical examination of tissues and their products, animal experiments, necropsy and microscopic studies. Life is

> "nothing but a sum of phenomena submitted to the ordinary physical and chemical (that is, mechanical) laws. To look for the reasons of these laws is 'transcendental pertness.' . . . Life is essentially cell activity and the cell the basic unit to be studied."

And forthwith he challenged the antiquated humoral theories of Carl von Rokitansky, the unquestioned leader in pathological anatomy of the times. In 1847 Virchow with his friend Benno Reinhardt, in the divine assurance of youth, brought out the first volume of the *Archiv für pathologische Anatomie und Physiologie und für klinische Medizin* (note the inclusion of clinical medicine) which he was to edit until 1902 when volume 169 appeared.

When a great epidemic of typhus broke out in Upper Silesia in the winter of 1847–8, Virchow and Barez were sent to report on this public scandal. Their plea for education, freedom, and prosperity for the Polish minority upset the rigid Prussian government. With the outbreak of revolution in Berlin in 1848, Virchow fought as a republican on the barricades, and again demanded medical reform.* He was suspended in 1849, but reinstated on probation as prosector though deprived of free board and lodging at the Charité. But the powers of reaction gradually prevailed and eventually he was silenced. He took the only way out and in November, 1849, moved to Würzburg as Professor of Pathology. Soon afterwards he married Rose Mayer and settled to a life of teaching and research. Friendship with congenial colleagues followed and his fame mounted, bringing him many brilliant pupils, among them Rindfleisch, Hoffman, Klebs, His Sr., Gegenbaur, E. Haeckel. About this time his views on "cellular pathology" crystallized out, and in an editorial to the eighth volume of the *Archives,* in 1855 when he was 34 years old, he first used the famous words "Omnis cellula e cellula."

In 1856 Virchow accepted the Chair of Pathological Anatomy in Berlin, largely because of the influence of Johannes Müller, and the offer of a special Institute at the Charité.

*His illustration of the theory of disease, "I know a family—a very exalted one—in which the grandfather had softening of the brain, the son hardening of the brain, and the grandson no brain at all" was well understood in Germany to refer to the three kings of Prussia. Frederick William the Second, Third, and Fourth. Virchow made little secret of his contempt for the national religion. He was an agnostic and denied the existence of a hell and the devil, of whom he said "only a benighted Mecklenburg parson could be so foolish as to believe in him." (Semon, 1902.)

Dr. Samuel D. Gross, the eminent American surgeon, visited the Institute in 1868 and tells us that the laboratory

> "is a model in its way, admirably adapted to the wants of the student for improvement in the use of the microscope and the examination of morbid specimens, which are passed round in jars or on plates on a neat little railway carriage, so that every one may have a full opportunity of inspecting them. Microscopes are provided in great numbers. . . . Virchow is a most patient and laborious investigator, and yet he never seems to be in a hurry. His dissections seldom occupy fewer than two and a half to three hours each. Every organ of the body is thoroughly explored. For years past his habit has been to open, every Monday morning, a cadaver in the presence of his private pupils with a view of instructing them in the art of conducting autopsies—holding the knife, using the saw, and taking notes, the whole being supplemented by microscopic inspection of the more important diseased structures. . . . A work from his pen on the subject of autopsies was lately issued at Berlin. On Wednesdays and Saturdays he devotes two hours before his class to the illustration of pathological anatomy."

The first assistants were Hoppe-Seyler, the great biochemist, and von Recklinghausen. Pupils flocked to him from far and wide, and the next few years saw the publication of some of his greatest works. Supreme among them is the "Cellular Pathology, based on physiological and pathological histology" which appeared on August 20, 1858. Based on a series of lectures given early that year to Berlin medical practitioners, it sums up what he had been thinking and writing over the previous 12 years. Of this work Lister said that it had

> "established the true and fertile doctrine that every morbid structure consists of cells which have been derived from pre-existing cells as a progeny. . . . Even those morbid structures which deviate most from the normal structures are known to be derived as a progeny from normal tissue—from normal cells, driven to abnormal development by injurious agencies."

These principles are developed brilliantly in Virchow's other great work, *Die krankhaften Geschwülste,* published between 1863 and 1867.

For us in England it is pleasing to record Virchow's staunch friendship for our own country. He had been brought up at a time when Europe felt strongly the reaction that followed the Napoleonic Wars and England alone stood for political freedom. He had seen, at close quarters, a bureaucratic and feudal régime in action in Prussia, and had suffered for the part he had taken in the revolution of 1848. Virchow made many close friends in England, amongst them Huxley, James Paget, Lister, Pye-Smith, Kanthack,

and Shattock. He was well informed about British medical writings and he frequently acknowledged the great services of John Goodsir of Edinburgh in the creation of cellular pathology as a discipline. Felix Semon, who knew him well and has written down, with devastating frankness, his weaknesses and oddities, says that the warmth of his receptions in England filled him with deep gratitude. When he gave the Croonian and Huxley Lectures in London he experienced a triumphal progress which almost overwhelmed him.

It is surely right and proper that we pause for a few moments to recall with gratitude and respect Virchow's great achievement given to the world just one hundred years ago. What this has meant to medical science you will learn from the distinguished contributions that follow. May I conclude by quoting a moving remark of Socrates.

> "Although my mind is far from wise, some of those who come to me make astonishing progress. They discover for themselves, not from me—and yet I am an instrument in the hands of God."

Fortunate, indeed, is he about whom this may be said.

In the preparation of this paper I have received much help from many friends, especially from Dr. Helmut Drubba of Essen, Dr. Walter Pagel of London, and the Cultural Attaché of the German Embassy, London.

Figs. 1–4 are reproduced by permission of the British Museum, and Figs. 5–9 by permission of the Landesbildstelle, Berlin.

I have drawn fully upon the authoritative works of the following and upon innumerable shorter papers in British, American, and German journals.

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DIAGNOSIS FROM CELLS

BY

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"When we recognize what our teachers and leaders accomplished without present-day methods, we will do well to bow our heads deeply before them."—Gierke (1884).

The methods used by Virchow which led to his work on cellular pathology are more akin to those of the cytologist than the morbid anatomist. The year 1858 is also notable for the first satisfactory staining of a histological section by Joseph von Gerlach (Fig. 1), as the result of a fortunate accident (Gerlach, 1858a and b). One evening, thanks to an oversight, he left cerebellum fixed in potassium bichromate in a very dilute solution of ammonia carmine. The following day the sections showed much better differentiation of nerve cells and fibres than had ever been seen before. Twenty-five years later Gierke wrote a history of staining to celebrate Gerlach's discovery. Gierke believed that Gerlach was the first to stain a section, although so much good work had been done earlier that now it is not easy to say which advances actually came from Gerlach's discovery.

Earlier work of great value included that of Corti, who in 1851 wrote "Reserches sur l'organe de l'ouie des mammifères." In 1854 Hartig (1854, a, b, and c) found that the nucleus of a cell stains only after it is dead, and that the granules of chlorophil have an affinity for carmine, and in 1849 Goeppert and Cohn used carmine and madder to make cell contents more visible. Hill's (Fig. 2) remarkable work (1770) included the use of maceration, fixation (spirits of wine), mordants (alum, sugar of lead), metallic impregnation (quicklime and orpiment in succession to stain stems brown), and stains (cochineal and boiling green sealing wax). Finally the greatest credit must go to Anthony van Leeuwenhoek (1719), the discoverer of the microscope, who compared the muscles of a fat and a thin cow after staining with crocus (saffron) macerated in spirits of wine. He tells us that he prepared very thin sections so that they were quite transparent. This experiment was described in a letter to the Royal Society dated August 21, 1714, but was not published for five years. It is therefore more accurate to say that Gerlach was the first to popularize histological methods in morbid anatomy.

Diagnostic cytology is the senior science. Virchow and his pupils used both cytology and histology, and a number of eminent pathologists, including Dudgeon and Ewing, continued this habit until recent times. The majority of pathologists, however, abandoned cytology and even denied it any place in diagnostic methods. So Dürck (1904) wrote:

> "Hence there is no such thing as a 'carcinoma cell' or a 'sarcoma cell'; the type of the tumour, whether it be epithelial, connective tissue, or mixed, can be determined positively only by the arrangement of the cells with regard to one another. For this reason extreme caution is necessary in giving an opinion on the character of a tumour from an examination of isolated tumour-cells."

Gerlach, however, found no opposition between cytology and histology, for to him they were different ways of looking at the same problem.

Before considering the remarriage of cytology and histology which began with the publication of the monograph by Papanicolaou and Traut in 1943, we should look at the work of some of those in England and the United States who kept the study of cytology alive during its dark days.

Lionel S. Beale (Fig. 3) was professor of medicine at King's College Hospital, London. In 1854 he wrote an outstanding book, *The Microscope in Medicine*, of which a fourth edition appeared in 1878. In it he states:

> "The people want the practical doctor, not the person who always has his eye at the end of a microscope, or is continually fishing for new facts in the excretions of the sick. . . . Nevertheless, the opinion that the nature of disease should be thoroughly investigated seems to be gaining ground. . . . But surely it would be right if rich bodies like Guy's, Saint Thomas's, and Bartholomew's took the lead in this matter. . . . I fear it will be difficult indeed to convince the authorities who have command of the purse."

Beale thought a pathologist could be obtained for £100 p.a. King's College appears to have been the only hospital with a side-room laboratory. It owed that entirely to Beale, and his book is the outcome of lectures delivered there in 1853. Beale gives a full account of the methods then in

FIG. 1.—Joseph von Gerlach (1820–96), Professor of Physiology and Anatomy in the University of Erlangen. (Photograph given by Professor K. Fr. Bauer, Director of the Institute of Anatomy, University of Erlangen.)

FIG. 2.—Sir J. Hill (1716–75), from the mezzotint by Houston after Coates. The impression is in the British Museum.

FIG. 3.—L. S. Beale (1828–1906), from an original photograph in the possession of and copyright by the Wellcome Historical Medical Library.

use for examining tissues. They were teased out in water and other fluids with the aid of a dissecting microscope. Frozen sections were usually cut with the help of nitrous oxide capsules, which were preferred to carbon dioxide snow because they were in ready supply for dentists. Later ether and ethyl chloride were used for freezing. Staining was at first with ammonia carmine, and Beale credits Gerlach with being the first to use this. Hardening and preserving agents used included alcohol, glycerine, carbolic acid, chloride of calcium, and chromic acid. Mounting was in gum damar or Canada balsam, but aqueous mounts were also used. It is most unfortunate that Beale's observations on discharges from the uterus and vagina have been forgotten. He knew of the *Trichomonas vaginae* described by Donné (1836) in cases of leucorrhoea, a discovery which made Donné the first to describe a living organism in pathological conditions. Beale appears to have agreed with those who considered this parasite "merely a cell of ciliated epithelium from the uterus." This is indeed curious because Beale insisted on the examination of vaginal mucus in a pure state and gives full instructions for warming slides. "In cases of cancer of the uterus, we should expect to meet with cancer cells in the discharges." He illustrates uterine squamous carcinoma cells (Fig. 4) in the urine of a woman with advanced carcinoma, and describes the findings in adenocarcinoma: "Villous processes, with loops of vessels, and the modified epithelial coverings." He also describes the "modern" scrape technique:

> "In many cases it is not difficult to remove a little of the softened cancerous matter upon the extremity of the sponge used in vaginal examinations. In this way a much better chance of meeting with entire cancer cells is afforded than by examining the urine."

He gives a drawing of cancer cells in the sputum (Fig. 5). Perhaps Beale used his microscope too freely, for giant cells and non-nucleated red blood cells made him disbelieve much of the cell theory. He "proved" in demonstrations to the Royal College of Physicians in 1861 that cells arise when the outer part of the primitive mass of "bioplasm" dies, so giving rise to the cell membranes.

About the year 1925 Professor L. S. Dudgeon (Fig. 6) at St. Thomas's Hospital, London, began to take an increasing interest in cytology. Cells, both innocent and malignant, from tissues and organs in various parts of the body were carefully studied. In collaboration with Patrick (1927) he published a paper on "A New Method for the Rapid Microscopical Diagnosis of Tumours," and later (1934) with Barrett on "The Examination of Fresh Tissues by the Wet-film Method." In this second paper the method was shown to be applicable to the study of pathological processes in general. Dudgeon and his colleagues achieved considerable success with the wet-film method, and the results obtained in the diagnosis of tumours compared very favourably with those from examination of paraffin sections.

A simple technique was employed. The tissue removed at operation, fresh and unfixed, was incised as soon as possible and the cut surface scraped with a scalpel. A film was made from the juice so obtained, and fixed immediately while still wet in Schaudinn's solution, stained with haemalum and eosin, and mounted in Canada balsam. The whole process could be completed in 10 to 15 minutes, and the intimate details of the structure of the nucleus and cytoplasm were shown far more clearly than in paraffin sections.

This method of examination for the detection of malignancy was obviously capable of wide application. It could be used in the operating theatre instead of a frozen section for the examination of a biopsy specimen, and in the out-patient department in the diagnosis of lesions of the skin or mouth. It was Dudgeon's custom to examine a wide variety of specimens by this method—sputum, faeces, pleural fluid—and on one occasion, in examining a film of sputum, he observed under the microscope a clump of cells which he immediately recognized as malignant. With the collaboration of C. H. Wrigley (1935) he instituted, for the first time, a systematic examination of sputa from cases of suspected lung cancer and demonstrated that malignant cells could be identified in a large proportion of the cases afterwards shown to be malignant, a procedure since confirmed by many workers in different countries. He then began to turn his attention to the examination of pleural and peritoneal fluids, stomach washings, etc., but his untimely death at the outbreak of the second world war unfortunately put an end to these activities.

Cytology and frozen section are not really rival methods for giving the surgeon a rapid answer when the patient is on the operating table. They reveal different aspects and ideally should be used together in every case. Cytology gives an excellent high-power view of the tumour cells but does not reveal their behaviour. Frozen section gives a somewhat crude low-power view, good enough for the accurate diagnosis of nearly all breast lesions. It shows the type of tumour and

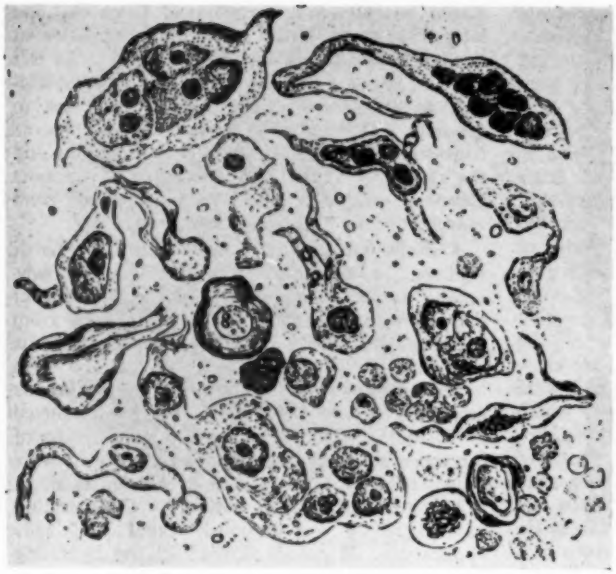


FIG. 4.—Beale's illustration (1878) of cells desquamated and found in the urine from a case of cancer of the uterus. Forms similar to these are found in smears from patients with squamous-celled growths.

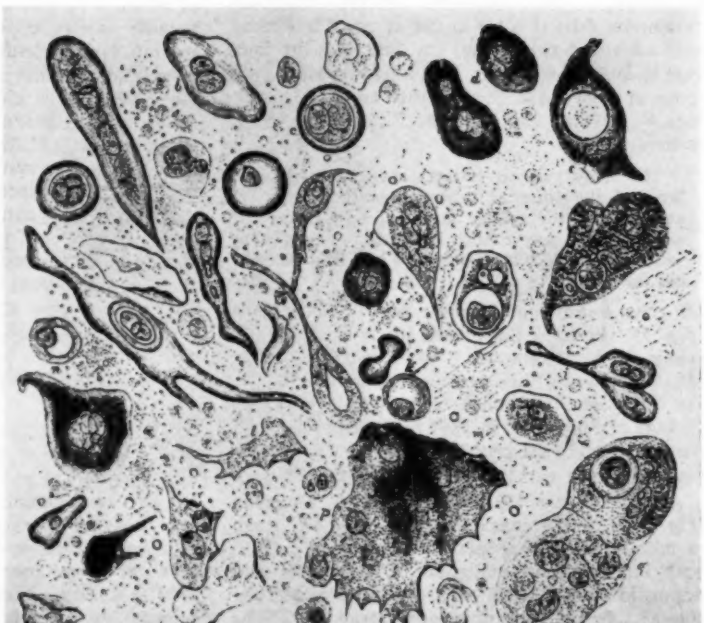


FIG. 5.—Beale's illustration (1878) of cancer cells in the sputum. Although working 20 years after Virchow he has not fully accepted the cell theory. His caption reads:

"Various forms of the so-called cancer 'cells' in sputum. Many of these bodies are clearly not 'cells,' but fragments of a mass containing nuclei, or small masses of bioplasm irregularly scattered through it. At *p* is represented a piece from the lower part of which the nuclei and portions of the mass surrounding them have been broken off, leaving cup-shaped cavities in which they were lodged."

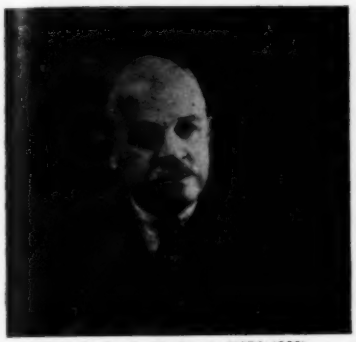


FIG. 6.—L. S. Dudgeon (1876–1938).

FIG. 7.—G. N. Papanicolaou (born 1883).

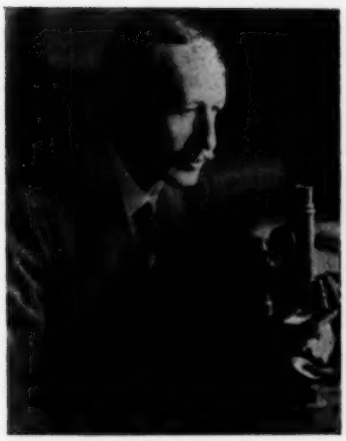


FIG. 8.—James Ewing (1866–1943).

its invasive properties, but fails when high-power detail is necessary. The method of frozen section was used freely and successfully by E. H. Shaw (1910, 1923), who reports more than 1,000 cases and first aided a surgeon in this manner in Yeovil, Somerset, in 1899. Shaw took under five minutes to make his diagnosis, the same time as is required to-day.

Those of us who practise both histology and cytology have no difficulty in seeing in every case how the appearances in the latter are a consequence of the former. The great oncologist, James Ewing, of the Memorial Hospital, New York (Fig. 8), stimulated the study of aspiration biopsy, and papers on this subject were published from the Memorial Hospital by Martin and Ellis in 1930 and 1934. For rapid diagnosis smears were made and studied cytologically. Fragments of tissue were fixed and sectioned. These are the methods applied now with such success to bronchial mucus and other fluids. Thus from the early 1920s onwards Ewing saw how these two sciences were correlated and could argue from one to the other in a way present-day pathologists should try to copy. Ewing endured ill-health all his life and diagnosed his terminal illness by the disciplines he had himself developed.

When a young boy Ewing fell and broke his hip. This accident nearly proved fatal, and he suffered throughout his life from osteomyelitis and often had to have sequestra removed from various parts of the right hip; some of these pene-

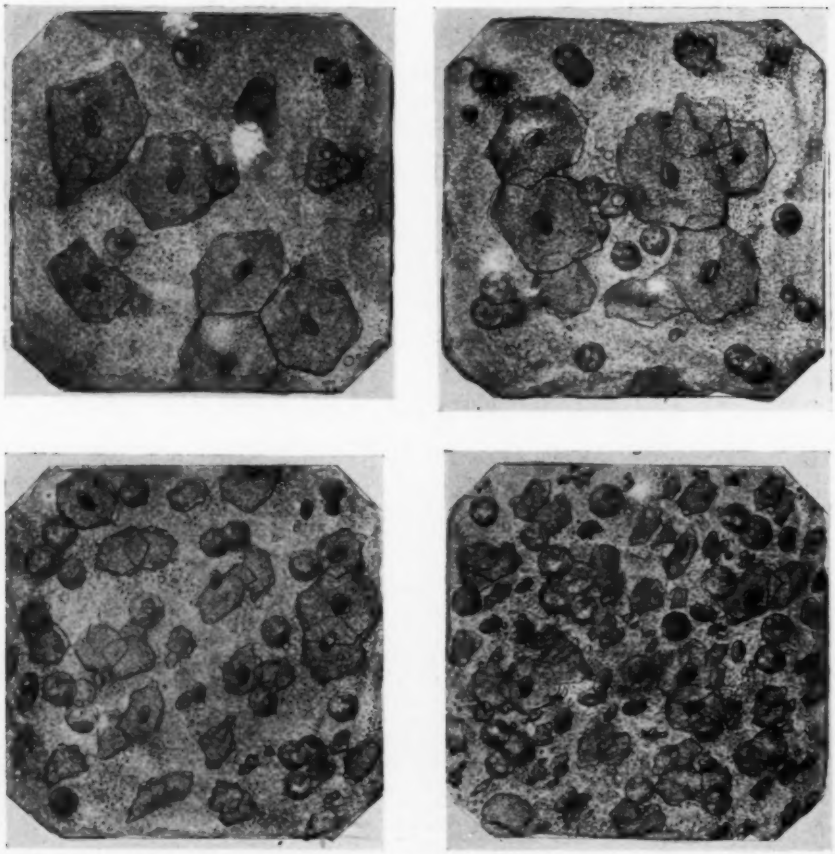


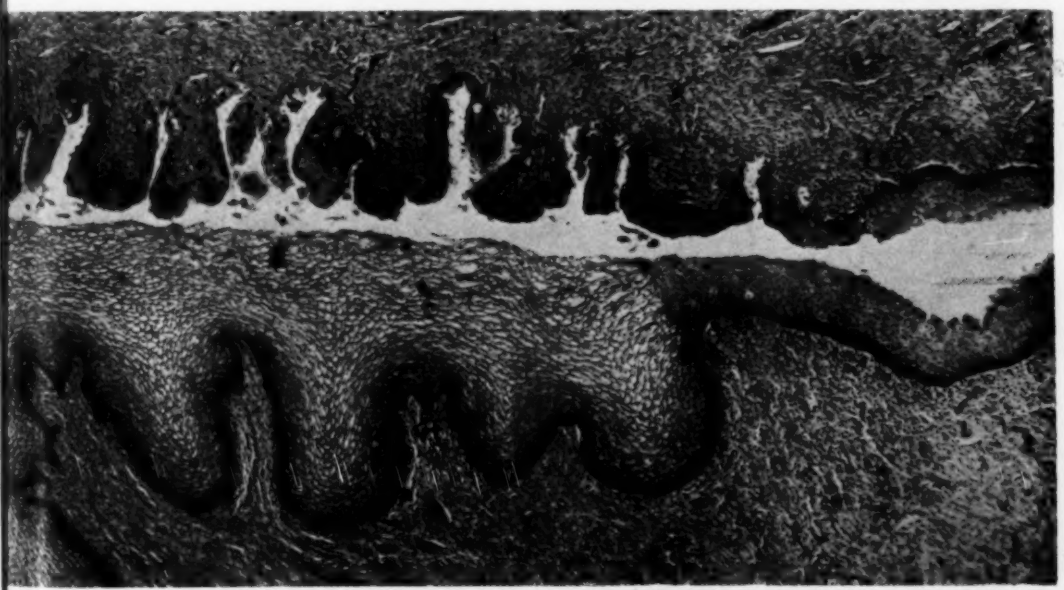
FIG. 9.—Drawings by Pouchet (1847) of the cytology of the intermenstrual phase in women. The first four figures represent the condition two, four, six, and 10 days after the cessation of menstruation.

trated the bladder. Five years before his death he developed vesical calculi which were removed by Dr. Roy B. Henline. Afterwards Ewing examined the centrifuged deposit of his urine many times and ultimately found cancer cells on three occasions. Prostatic cancer was suspected, but Ewing knew the cell he was looking at, and cystoscopy before a contemplated prostatectomy showed a small fundal carcinoma. At operation the bladder wall was too friable for satisfactory removal of the carcinoma and Ewing was returned to his bed. Two days later Dr. Stewart, pathologist to the Memorial Hospital, knew the highly malignant nature of the tumour. He discussed this with Dr. Ewing Jr., and they decided that when Ewing asked to see his section they would substitute one from a much less malignant bladder tumour. Two days later he asked for his slides and the false ones were shown him. His son was in the room at the time. After looking at the sections for two or three minutes Ewing said: "Boy, what are you trying to do to me? You must be naive to send me these sections. They are not mine. I know, because I have already seen the type of cell associated with my type of bladder cancer. Now go and get me my own slides." Dr. Ewing Jr. then says: "Of course, the only thing Dr. Stewart and the rest of us could do was to hand him the proper sections. After looking at them, he was well satisfied." The cytological diagnosis he had made during the preceding eight or 10 months corresponded to the histology.

Papanicolaou's (Fig. 7) interest in cytology arose from a study of the cyclic changes in guinea-pigs (Stockard and Papanicolaou, 1917). Earlier studies (Fig. 9) along the same lines, both macroscopic and microscopic, had been made by Pouchet in 1847 in Rouen. Allen and Doisy (1923) used this oestrogenic response of vaginal epithelium to isolate the oestrogenic hormone from the fluid in ovarian follicles. In 1923 Papanicolaou extended these animal studies to women at the Women's Hospital of New York City and before long realized that he could recognize carcinoma cells. He reported these studies in 1928. Unfortunately the general opinion then was that these cytological findings were superfluous and he abandoned the work for further studies on some of the sex hormones (Papanicolaou and Shorr, 1936; Papanicoloau, Ripley, and Shorr, 1939; Papanicolaou, 1955). Later the studies on cancer of the uterus were resumed more exhaustively with Traut, and the result of three years' work was published in 1943 in the monograph which began the world-wide acceptance of gynaecological cytology although the opposition of large numbers of pathologists did not end with this publication. Opposition has now almost disappeared as a consequence of the good results obtained in practice by centres scattered widely over the world and the demonstration of a histological basis.

The development of a histologically unjustifiable nomenclature is one of the unfortunate consequences of the opposition to cytology by histopathologists. When stimulated by oestrogens the stratified squamous epithelium of the cervix and vagina responds by deposition of glycogen in the cytoplasm of all cells except the deepest and the nuclei then become small, central, and pyknotic. Many cytologists are now calling this process cornification. True cornification is often found in the cervix and vagina in women with prolapse. It begins with the development of a stratum pigmentosum and stratum corneum over the capillary loops of the corium and in more advanced lesions these patches coalesce. Squames shed from the cornified patches naturally have no nucleus. On page 238 of their 1917 paper Stockard and Papanicolaou describe them correctly:

> "There are, however, to be seen, particularly toward the end of the first stage, a certain number of elongate, cornified cells without nuclei, which are desquamated from the more external parts of the vagina."



IG. 10.—Section of the cervix and vagina near the fornix of a newborn baby. An unusually large papillary erosion is present (upper left); below this is the typical many-layered stratified squamous epithelium of full oestrone action; to the right (upper and lower) is shown stratified squamous epithelium which is oestrone resistant. It has failed to develop the glycogen response; there is a sharp dividing line between the oestrone-sensitive and resistant epithelia not unlike that seen in carcinoma *in situ*. This oestrone-resistant epithelium is commonly but inaccurately referred to as metaplastic. The cells desquamated from it can lead to false diagnoses of oestrone deficiency. This appearance is rare in the newborn but common in later life. Haematoxylin and eosin. × 40.

Basal cells cannot desquamate naturally and are not found in vaginal pool smears. The cells many cytologists call basal are from an epithelium inadequately stimulated by oestrone, so histologically they appear different from those of the fully stimulated epithelium. Embryologists and gynaecological pathologists are familiar with many varieties of squamous metaplasia and oestrone-resistant squamous epithelium in the cervix and vagina, but many of these appearances are little understood and are inadequately considered cytologically (Fig. 10).

Histology becomes a much more interesting subject when it is correlated with cytology. This relationship will be considered more particularly in the bronchial mucus and in carcinoma *in situ* of the uterus. It is important in all regions, however. For the stomach Schade's (1956) studies of chronic antral gastritis should be referred to. The best pre-operative method of diagnosing carcinoma of the prostate is cytological, and the findings after stilboestrol therapy have a histological basis (Peters and Franck, 1952; Bamforth, 1953; Fergusson and Gibson, 1956). Research is needed to obtain more consistent results when tumours of the bladder are present, and there is no field in which it is more important for the cytologist to be familiar with the histology of the lesions. Breast cytology can be dangerously misleading if reported on by someone not skilled in breast histology.

TABLE I
COMPARISON OF SPUTUM, TRAP SPECIMEN, AND BIOPSY (174 CASES)

	Positive	Negative	Not Done
Sputum	21	83	70
Trap cytology	54	96	24
,, histology	62	58	54
Biopsy	57	38	79

Sputum positive, other investigations negative	5 cases
Trap specimen positive, other investigations negative ..	16 ,,
Biopsy positive, other investigations negative	13 ,,
25 known cases of cancer of the lung among the 85 negatives:	
Sputum and trap specimen examined	2 ,,
Trap specimen only examined	6 ,,
Trap and biopsy specimens examined	2 ,,
Sputum, trap, and biopsy specimens examined ..	15 ,,
50% with cancer proved by biopsy alone	
78% with cancer proved by using all methods	

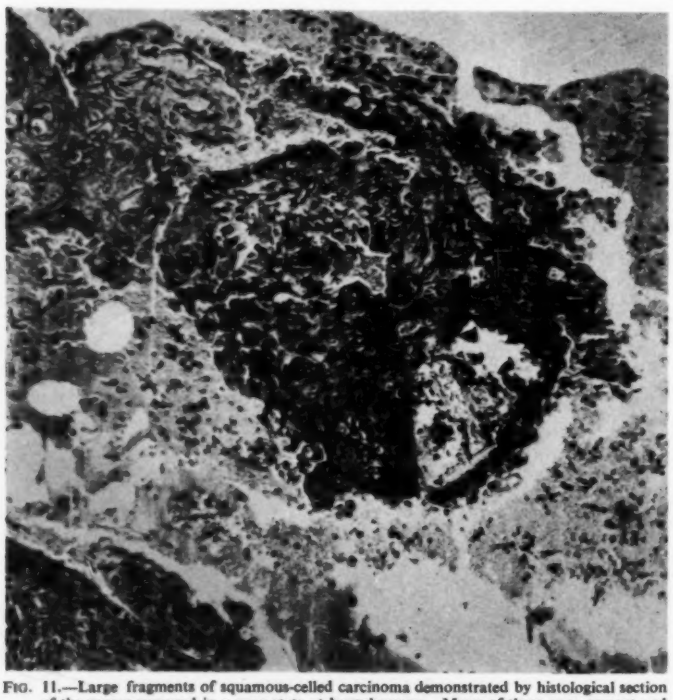


FIG. 11.—Large fragments of squamous-celled carcinoma demonstrated by histological section of the mucus removed in a pure state at bronchoscopy. Many of these were present and practically all were diagnostic and better than the fragments obtained by deliberate biopsy. This must be considered superior to the cytological method, but it is not recommended as a screening method for the sputum. Haematoxylin and eosin. × 150.

For various reasons the results with sputum cytology at the Derbyshire Royal Infirmary (G.R.O.) are not as good as those reported by Dudgeon and Wrigley in 1935, but because of the dramatic results obtained at regular intervals we feel the service must be offered to the clinicians in spite of many disappointments. The results with bronchial mucus are much more satisfactory, and nowhere has the ability to command histological methods as well given such a rich reward. Table I shows that bronchial biopsy alone gave the diagnosis in only half the patients with bronchogenic carcinoma, but the figure was raised to 78% by adding a full study of the bronchial mucus.

The bronchial mucus must be obtained in its pure state by the thoracic surgeon, and because of its method of collection it is referred to as the "trap specimen." It varies

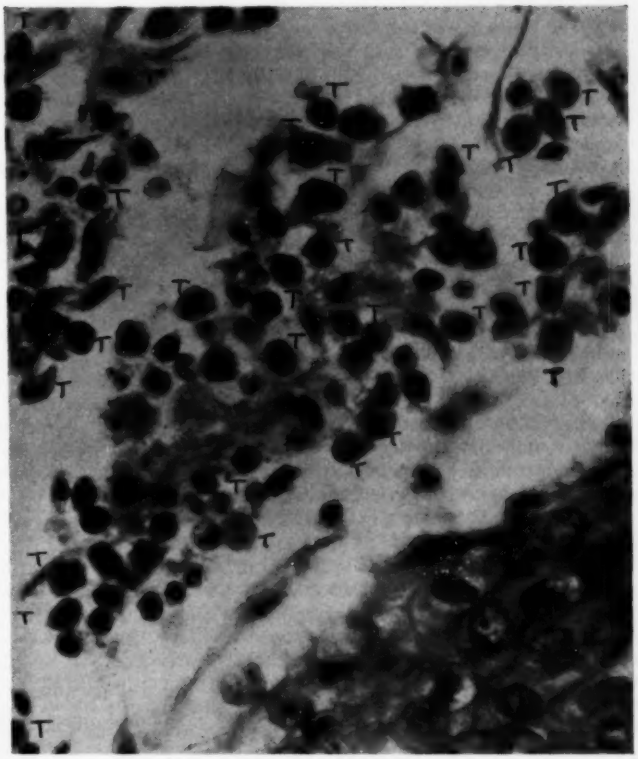


FIG. 12.—Innumerable *Trichomonas vaginalis* demonstrated in the histological section of the vagina of a baby dying at 27 days. The elliptical nucleus is well shown in many of the parasites (marked T). The stratified squamous epithelium of the vagina is seen in the lower right part. Haematoxylin and eosin. × 600.

in amount and must never be diluted with saline. At first it was examined cytologically only; but quite early, rather than waste the residue, some was fixed in Zenker's solution and studied histologically. In many cases the results of the histology of the trap specimen are extraordinary, numerous perfectly preserved carcinoma fragments being present (Fig. 11). The results in these patients were so very good that it was thought there was no need to take biopsies. However, when the full series was studied it was found that in 13 cases with a positive biopsy there was a negative result for the trap specimen. The opposite finding of negative biopsy and positive trap specimen was verified 16 times. The conclusion is not that one method is better than another, but that most diagnoses are proved when all methods are employed. Some observers have better results than these, but we do not expect to be able to diagnose all cases of carcinoma of the lung.

Excellent results follow the use of all methods in cancer of the uterus. A review of the past 463 cases of invasive carcinoma of this organ in Derby shows that by one means or another all the patients were diagnosed. There is no known case in which inspection by the gynaecologist, biopsy, and cytology all gave negative results. Some women had negative results to one or two of these methods but none to all three. These results with photomicrographs are given elsewhere for both the bronchial mucus and the uterus (Osborn, 1956a and b; 1958).

Table II shows the Derby cases of carcinoma *in situ* of the uterus. In the majority the first stage in the diagnosis was cytological. Some had negative smears, but the main reasons for this were poorly taken smears and a very small or incompletely developed lesion. Some pathologists would call the appearances those of basal hyperplasia and others carcinoma *in situ*. The average

TABLE II

AGES OF 87 CASES OF CARCINOMA *IN SITU* OF CERVIX

	20s	30s	40s	50s	60s	70s	80s
87 *in situ* ..	13	26	27	16	4	1	0
150 invasive ..	3	17	33	51	32	13	1

Average age 39 years 11 months, range 22–72 years; of invasive 54 years, range 27–83 years

A, Preliminary cytology only	10 cases
A, B, Preliminary cytology and biopsy	67 ,,
B, Preliminary biopsy only	3 ,,
C, Operation specimen only	7 ,,

CYTOLOGY OF THE 77 CASES

39	cancer cells
19	,, ,, probably present
6	,, ,, possibly present
17	,, ,, not present

Six cases in transitional stage of basal hyperplasia to carcinoma *in situ* all had negative cytology.

Two cases were of the cervical stump after subtotal hysterectomy.

TREATMENT

Hysterectomy	55 cases
Radium	9 ,,
Radium and operation	3 ,,
Amputation of cervix	3 ,,
Observation and cautery	1 case
Observation	16 cases

age of these women is 14 years less than the average for those with invasive squamous-celled growths, thus giving a long time for diagnosis in the gynaecological clinics. It is a great pity that we are still not diagnosing the majority of these cases which are in women not attending hospitals or in women attending hospitals lacking a service in cytology. Because these women should all remain well there can be no greater service rendered by cytology. The condition is fully considered elsewhere (Osborn, 1958).

It is impossible to consider the value of cytodiagnosis in all branches of hospital work, as it has given rise to unexpected findings in many fields, so two will be mentioned in conclusion. A number of venereologists have reported that infection with *Trichomonas vaginalis* disappears at the menopause. The best method of diagnosing this infection is cytological. By this means it was known that in the Derby gynaecological clinics the maximum incidence is about the time of the menopause and cases have been recognized in women in the 70s and 80s. Mr. H. R. M. Richards was, therefore, asked to record the age incidence in the V.D. Department, and he found a maximum incidence around the age of 20 (Osborn, 1956b). The young women are not used to attending gynaecologists, because, knowing how the infection has arisen, they mostly go to the V.D. Department. The full picture of this disease is not obtained from one hospital department. The second unexpected result of interest was in a baby girl dying on the 27th day of life. It is customary to examine the uterus and vagina in all neonatal female deaths to see the range of epithelial appearances (Fig. 10) and to obtain information on congenital erosions. The section from this baby girl (Fig. 12) showed great numbers of *Trichomonas vaginalis* in the vagina. Smears from the baby's mother were obtained and, as expected, were shown to contain *Trichomonas vaginalis* also.

What then should be the place of diagnosis from cells to-day? It is the same as it was 100 years ago, and has been in a limited number of laboratories since then. It is only one way of looking at a diagnostic problem, but when correlated with other diagnostic methods it is always interesting and often of the greatest value, and may lead to the correct diagnosis of cancer when inspection and biopsy fail. Cytology should therefore not be confined in a special department of its own but should be practised in the general laboratory. If cytology reveals some of the limitations of inspection and biopsy, these in their turn reveal some of the limitations of cytology, and most diagnoses are made when all methods are used.

We are greatly indebted to Mr. T. J. Shields, Librarian of the British Medical Association, and to Dr. F. N. L. Poynter, Librarian of the Wellcome Historical Medical Library, also to Dr. G. N. Papanicolaou and Dr. James H. Ewing for their assistance. The photomicrographs were taken by Mr. J. S. Fayers.

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THE BACTERIAL CELL

BY

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Our approach to the bacterial cell depends on our particular interests. The geneticist finds it a most useful plastic tool for studying mutations and variations, transductions and transformations ; the chemist is interested in it as a source of enzymes of bewildering variety ; he also helps in identifying the various components of the cell by fractionation and detailed chemical analysis of bacterial cell masses ; the cytologist is mainly concerned with the anatomy of the cell, using modern staining and microscopic methods, including phase-contrast and electron microscopy ; the bacteriologist is interested in the cell's interaction with its immediate environment, whether that be living tissue or dead and decaying matter.

For clinical pathologists gathered together to do honour to Virchow, it would seem most important to discuss, if only in outline, our modern concepts of the anatomy and physiology of the bacterial cell.

Definition

Bacteria belong to the kingdom of Protista, which also includes plant and animal forms and has many resemblances to the blue-green algae. According to majority opinion at the present time, the bacterium may be unicellular or multicellular, with haploid nuclear or chromatin material occurring as a single body and dividing by transverse fission. Cell division depends on constrictive ingrowth of the cell wall and cytoplasmic membrane.

Let us start with a simple diagrammatic representation (Fig. 1) of the bacterial cell. It is enclosed in a cell wall from which there may be extruded flagella and fimbriae and may be surrounded by a capsule. The main function of the cell wall is to give form and rigidity and some protection to the functional cell or protoplast. The cytoplasm has a lining or membrane which acts as a selectively permeable osmotic barrier and contains within it many granules varying in size from 10 to 20 mμ, composed mainly of ribonucleic acid (R.N.A.). Various inclusion bodies, such as volutin granules, lipid granules, etc., may be contained within the cytoplasm, and we shall refer to these later. The nuclear or chromatin material has the chemical and staining reactions of desoxyribonucleic acid (D.N.A.), but cannot be regarded as a nucleus in the sense that we use the term for animal cells, since there is no nuclear membrane, no centriole, and no unequivocal evidence of division by mitosis.

Cell Wall

Although a rigid lining membrane for the bacterial cell, similar to that for the fungus mycelium, was postulated by Cohn in 1875, it is only since the introduction of electron microscopy that cell walls have been clearly demonstrable. From bacterial suspensions which have been mechanically or sonically disrupted, the cell walls can be separated from the electronically more opaque cytoplasm by high-speed centrifugation at 8,000 to 10,000 r.p.m., after first removing any intact cells at 2,000 to 3,000 r.p.m. It is less easy to get pure cell walls with Gram-negative than with Gram-positive cells, and mechanical disruption gives better results than sonic disintegration. The cell wall constitutes about 20% of the dry weight of Gram-positive cocci and as much as 45% for *C. diphtheriae*. The thinner walls of Gram-negative bacteria probably account for considerably less than 20% of the total weight. The thickness of the staphylococcal cell wall has been estimated at 15 to 20 mμ, while that of Gram-negative bacilli, like *Bact. coli* and salmonellae, is around 10 to 15 mμ and of *Mycobacterium tuberculosis* 23 mμ. The selective rigidity of the outer casing is demonstrated by the finding that the cell walls of rod-shaped organisms when specially prepared for electron microscopy retain their cylindrical form, whereas the protoplast assumes a spherical form when the cell wall is dissolved by lysozyme. Cell wall suspensions have a milky white opalescent appearance, and in the case of chromogenic bacteria there is no contained pigment which is associated with the small particles in the cytoplasm.

There is considerable variation in the chemical composition of the cell walls in different bacterial species, the main constituents being peptide-poly-

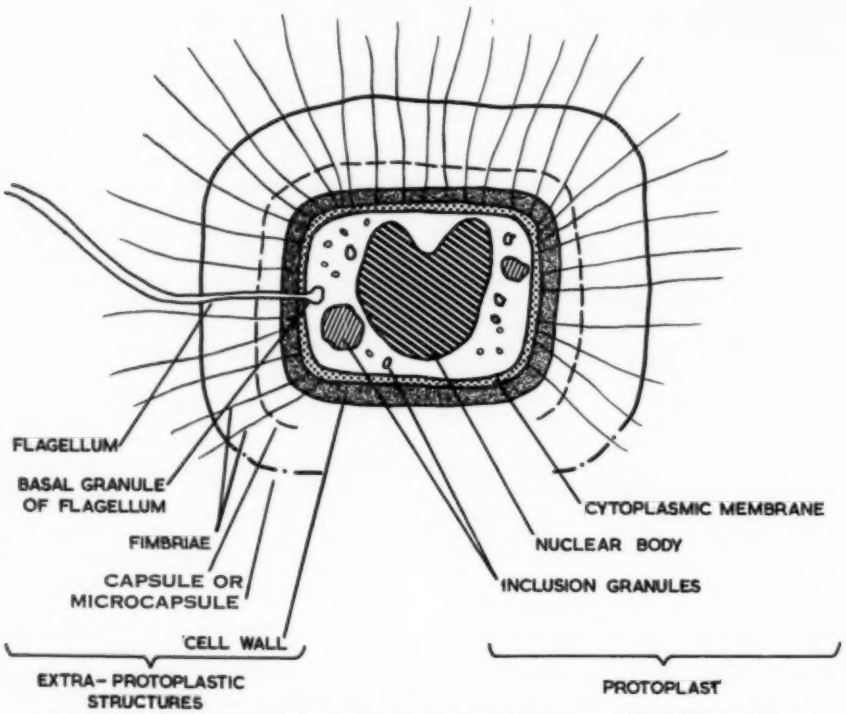

FIG. 1.—Diagrammatic representation of a bacterial cell (extracellular structures omitted at bottom of figure).

saccharide complexes. Certain major differences occur between the Gram-positive and Gram-negative organisms, the former having a limited range of amino-acids, whereas the Gram-negative bacteria have the same full range of amino-acids as have most proteins. The lipid content of the cell wall in Gram-negative bacteria is usually much greater than that of Gram-positive organisms (around 20% compared with 2–4%), and this high content of lipid may be related to the lipo-polysaccharide of the O antigen or endotoxin. Polysaccharides and hexosamine are present in about equal amounts in both Gram-positive and Gram-negative bacteria.

In the past few years more detailed analyses have been made of the chemical content of the cell wall and its possible precursors (see Park, 1958). Thus, analysis of staphylococcal cell walls has shown that muramic acid, D-glutamic acid, lysine and DL-alanine are present in a ratio of about 1 : 1 : 1 : 3, while examination of penicillin-inhibited staphylococci revealed an accumulation of a uridine-5′-pyrophosphate complex which could be a cell wall precursor, since it contained muramic acid and the amino-acids in the same ratio (Park and Strominger, 1957). These findings may be linked to the early suggestion by Duguid (1946), following observed morphological changes in penicillin-inhibited bacteria, that penicillin interferes specifically with the formation of the cell wall while allowing growth to proceed until the organism bursts its defective envelope and so undergoes lysis, and to the recent observations by Cooper (1954, 1955) that penicillin is specifically bound to a bacterial lipid fraction which could be the cytoplasmic membrane. At last we seem to be learning something about the mode and site of action of penicillin and perhaps of other antibiotics.

Another application of our knowledge of the chemistry of the cell wall concerns the action of lysozyme, which acts on a mucopolysaccharide with the release of N-acetyl hexosamine. When resistant variants of the susceptible *M. lysodeikticus* are obtained, chemical analysis shows no change in their sugar or amino-acid content but a 100-fold increase in the O-acetyl group. These

findings suggest that lysozyme resistance may be due to an ability of the organism to acetylate certain cell wall hydroxyl groups associated with the substrate, which in the sensitive strains are free and receptive (Brumfitt, Wardlaw, and Park, 1958).

Other lytic enzymes, such as that derived from *Streptomyces albus*, act on cell walls and can be used for preparing bacterial solutions for precipitation reaction, such as Lancefield grouping.

Capsules

Much controversy and confusion have arisen over the definition of bacterial capsules, and various alternative or additional names have been used, such as envelope, slimy layer, ectoplasm, and outer coat. We may define a capsule as a microscopically demonstrable slimy or gelatinous layer covering the cell wall and having a definite external surface. The term "envelope" is used by some bacteriologists if the external boundary is not sharp or the slime has a tendency to dissolution; others have described a "capsule membrane," but there seems no sound support for either of these two terms. A good deal of the confusion has arisen from difficulties in the demonstration of the capsule. The two most reliable methods are the wet-film India ink technique, used as a negative stain, and the specific antigen-antibody reaction erroneously called the Neufeld Quellung or capsular swelling reaction. Duguid (1951), who gives a detailed description of the India ink method, found that of dry-film staining techniques the eosin serum method of Howie and Kirkpatrick (1934) was the best. Recently, Novelli (1953) and others have used Alcian blue as a capsule stain, since it is the only dye so far recorded which will stain the bacterial capsule without previous mordanting. With one or other of these reliable methods, there is unanimity about the capsules of organisms like the pneumococcus, the anthrax bacillus, or Friedländer's bacillus. With *Pasteurella pestis*, on the other hand, there has been much divided opinion about capsule and envelope. Amies (1951) believed that the "envelope," dissolving in saline at 60° C., is no more than a particularly well developed capsule which can be shown to persist despite solvents. In addition to these demonstrable capsules, certain bacteria may carry surface antigens or "microcapsules" (Wilkinson, 1958) separable and antigenically distinct from the cell wall, for example, the M antigen of *Streptococcus pyogenes*, which can be dissolved off by trypsin, without affecting the cell wall or viability.

Tomcsik and his colleagues (see Tomcsik, 1956) have used the specific capsular reaction (a more correct term than capsular swelling reaction) to study the structural elements in the capsule and also as an indicator of the localization of surface antigens. When specific antiserum is added to capsulated bacteria, a precipitation reaction quickly occurs which renders the capsule visible without changing its size or shape. This reaction has been used in the identification of such bacterial species as pneumococcus, streptococcus, Klebsiella, Haemophilus, Neisseria, Pasteurella, and Bacillus, and has helped to show that the capsular substance of the anthrax bacillus is not a polysaccharide but a glutamic acid polypeptide, while that of *Pasteurella pestis* is also a protein or a protein-polysaccharide complex.

Flagella

Flagella are unusual in that they lie outside the cell wall and may be removed without killing the cell, yet have a characteristic movement which indicates that they are part of the living cell. Despite the ingenious artistry and arguments of Pijper, we may accept the flagella as locomotor organelles with their origin in a basal granule inside the cell wall. Structurally, they are non-tapering filaments in the shape of a cylindrical helix, which may have either one of two different wavelengths. The filaments vary in length up to 12 μ and are approximately 12 mμ thick. Chemically they are composed predominantly of protein made up of elongated molecules (molecular weight 41,000) with a characteristic amino-acid composition from which histamine, tryptophane, proline, and cysteine are lacking. The great variety of antigenic entities among flagella with very similar protein content suggests that these antigenic differences depend on the arrangement rather than the composition of the amino-acids; this plasticity may also account for the variation from specific to group phase which characterizes many salmonella flagella.

In stained preparations of flagellated bacteria the actual number of flagella is much greater than are seen *in vivo* under dark-ground or phase-contrast microscopy. This difference is probably due to a tendency for flagella to aggregate with any motion; aggregation into quite thick filaments is particularly well seen in the elongated forms of *B. proteus* grown on agar in the presence of penicillin (see Fleming, Voureka, Kramer, and Hughes, 1950). When flagella have been broken off from a young culture they regenerate within half an hour; on the other hand, there is no evidence that they wear themselves out or even become frayed by active movement.

Space does not allow any discussion of the interesting studies of Stocker and others on the genetic transductions of flagella and motility.

Fimbriae

Before we pass inside the cell wall mention must be made of another type of appendage, the short, slender and very numerous fibres, called fimbriae by Duguid, Smith, Dempster, and Edmunds (1955) and Duguid and Gillies (1957) to denote a fringe which surrounds many species of Gram-negative bacteria (*Bact. coli, Bact. cloacae,* Salmonella, *Shigella flexneri,* but not *Sh. sonnei* or *shigae.* They are about half the thickness of flagella, and fimbriation is enhanced by frequent passage in broth culture but has no relationship to motility. These fimbriae have strong adhesive properties and are responsible for agglutination of the red cells of various animal species. The fimbriae from different serological types of *Shigella flexneri* are antigenically similar and may be responsible for some of the non-specific agglutinins present in the blood in many individuals. It is still uncertain what function, if any, they serve.

The Protoplast

Separation of the cytoplasm from the cell wall was observed many years ago by Fischer (1900), who described as " plasmoptysis " the ejection of the cytoplasm from organisms like *B. anthracis* and *Vibrio proteus.* The empty casing either adhered to or was separated from the cytoplasm, which then assumed a spherical form. This phenomenon was recently rediscovered by Stähelin (1953, 1954) in weak-walled anthrax bacilli. The term protoplast was, however, first used by Weibull, who dissolved the cell wall of *B. megaterium* by lysozyme and maintained the spherical naked cell in a stabilizing solution of sucrose or polyethylene glycol for at least 24 hours. Attached flagella can be demonstrated in the intact cell although motility does not occur, probably for physical reasons. The protoplasts disintegrate rapidly in non-stabilizing solutions or if the suspension is aerated and the lysate is seen to contain " ghosts " or empty vesicles and small granules. The ghosts, which probably represent the cytoplasmic lining, are composed of delicate membranes and contain most of the pigmented material of the cell. The metabolism of protoplasts and whole bacterial cells is remarkably similar, so that, besides the cytochrome system, most of the cell's enzymic activity is contained within the protoplast. Phage production and spore formation also occur, provided these phenomena have been initiated before dissolution of the cell wall. To quote McQuillen (1956), who has carried out many interesting studies with protoplasts :

> " Intact bacteria and the protoplasts derived from them have closely parallel capabilities. Both forms respire ; both synthesize proteins and nucleic acids and form adaptive enzymes ; both can support the multiplication of virulent and temperate bacteriophages ; both can support the development of endospores ; both grow in appropriate media ; and both can divide. Moreover, many of these activities are carried out at approximately similar rates and to similar extents by the two forms, the whole and the part. And yet the part is not the whole ; there are differences in behaviour. To what extent some of these differences are due to the use of unsuitable conditions is not yet known."

Protoplasts cannot build a cell wall, possibly because a starter of cell wall material is needed before more can be laid down. Similarly, the failure to sporulate unless the process has already been initiated probably means that cell wall is needed to complete spore formation. Again, although phage will multiply inside the protoplast, this body cannot be infected with virulent phage, which seems to need the cell wall for initial penetration.

The Nuclear Material

Fierce arguments have been raging for some years on the nature and interpretation of the changes that are demonstrable in the chromatin bodies or nuclear material of the bacterial cell during the process of growth and division. The argument largely turns on whether the chromatin bodies which grow in number with the growth of the cell are composed of chromosomes which divide like the chromosomes of animal and plant cells, i.e., by mitosis, or whether they divide directly or amitotically, as happens with many of the higher bacteria and Protista. The opposing views are set out by Robinow and DeLamater respectively in their chapters in the *Symposium on Bacterial Anatomy* (1956). Robinow, using Feulgen and acid Giemsa stains, has studied particularly the development of the single chromatin body in germinating spores, and his interpretations of the observed phenomena carry convincing evidence for direct amitotic division. DeLamater has used the actively growing cell for his studies, and, having perhaps too readily assumed that bacteria divide by mitosis, interprets his findings to fit this hypothesis. He claims that both *B. megaterium* and *Bact. coli* have three chromosomes which can be shown pictorially to divide simultaneously by a complex mitotic mechanism inside the nuclear membrane. He and his colleagues also describe mitochondria and a centriole, which Robinow, on the other hand, regards as an acces-

sory chromatin granule. It is impossible for the layman to adjudicate on the merits of these divergent stories by highly specialized cytologists, but support can be given to DeLamater's viewpoint that the truth will best be elicited by the simultaneous application of other techniques, such as those of biochemistry and bacterial physiology along with the cytochemical methods of the cytologist.

If we turn for a moment to the evidence of the microbial geneticist, it is obvious that genetic exchange of nuclear material takes place as a result of sexual pairing in certain species; in addition, transfer of genes occurs to cause the phenomena of transformation in pneumococcus and haemophilus types and of transduction of toxigenicity in *C. diphtheriae* or motility in salmonella. The cytologists have so far been unable to contribute to a better understanding of these strange happenings, although there has been some correlation between cytological changes and genetic exchange in higher organisms like Drosophila and maize. There is little doubt that D.N.A. is the active principle in the genetic mutations of bacteria, mediated sometimes through bacteriophage. It has also been shown that genetic differentiation of the D.N.A. material is linear, i.e., the "bacterial chromosome" may be regarded as a long, thread-like D.N.A. molecule, carrying the genes in an orderly sequence like the inscription on a tape. The D.N.A. molecule, pictured by Watson and Crick (1953) as a cylindrical double helix with a diameter of 2.5 mμ, would correspond to a thread about 1,000 times the length of the parent bacterial cell. Attempts at cytological study of the D.N.A. molecule are now being made by electron microscopic examination of ultra-thin sections of bacteria. It is suggested that the thread is coiled up to form a tube, and in the photomicrographs of Maaløe and Birch-Andersen (1956) tube-like structures cut in all different directions can be demonstrated. It may be possible in the future to learn more about the nucleus and its mode of division by detailed studies of this kind.

Cytoplasm and Inclusion Bodies

In electron photomicrographs of sectioned bacteria (staphylococci, streptococci, paracolon bacilli, aerobacter, mycobacteria) the cytoplasm seems to consist of fine granules of 10 to 20 mμ with sometimes larger granules, the total number in a single cell being of the order of 10^4 to 10^5. Chemically the cytoplasm is composed mainly of ribonucleic acid and protein and contains most of the enzymic activity of the cell, partly in its membrane. The small size of the granule and the multiplicity of enzymes in many bacteria suggest that there will be numerous enzymically different types of granules. They are probably derived from the nucleus, growing by the formation or accumulation of enzyme proteins. In discussing the relationship of bacterial cytoplasm to that of other lowly creatures, Bradfield states that :

> "the most striking structural difference between bacterial cytoplasm and the cytoplasm of animals and plants is the absence from bacteria of all kinds of intra-cytoplasmic membranes; the oxidative granules are not wrapped in membranes to form mitochondria; the RNA-rich granules are not arrayed on membranes to form endoplasmic reticulum; and finally the cytoplasm as a whole is not separated from the nucleus by a nuclear membrane."

Among inclusion bodies, the most commonly seen are the Babes-Ernst metachromatic bodies, often called volutin granules. These metachromatic granules, seen best in *C. diphtheriae* and ranging in size up to 0.6 μ, are very electron opaque and chemically contain polymetaphosphate and probably other phosphorus compounds. Smith, Wilkinson, and Duguid (1954) found that, although volutin granules might account for 20% of the bacterial volume, only 1% of the dry weight was polymetaphosphate.

Wilkinson and his colleagues (Williamson and Wilkinson, 1958; Macrae and Wilkinson, 1958) have also studied the lipid inclusions found particularly in the larger varieties of the Bacillus species. These lipid granules, which are composed mainly of a polymer of β hydroxybutyric acid, are most numerous in cells about to sporulate or when grown in semi-synthetic culture media containing an excess of glucose, pyruvate, or β hydroxybutyrate as the main carbon sources for metabolism. They are regarded by the Edinburgh workers as stores of carbon and energy and are probably used up in the process of sporulation. Whether inclusion bodies like volutin and the lipid globules serve a specific function in cellular metabolism, or whether they represent an abnormal or pathological state of the bacterial cell, is a debatable point. As already mentioned, the pigments of chromogenic bacteria are found predominantly within the cytoplasm, and Schachman, Pardee, and Stanier (1952) have observed many well-defined chromatophores of approximately 40 mμ diameter in the cytoplasm of *Rhodospirillum rubrum*. They were absent from organisms grown in the dark. Probably many other photosynthetic bacteria possess similar chromatophores.

Osmotic Activity of the Bacterial Cell

Having discussed some aspects of bacterial anatomy, we may conclude with a reference to the bacterial cell's mode of living. As with other cells, the active life and growth of bacteria depend on an interchange of nutrient and waste products between the interior of the cell and the external environment. This involves a selective intake of nutrient molecules and the expulsion of waste molecules, while the vital enzymic and nuclear substances of the cytoplasm are retained. There is good evidence that the cytoplasmic lining membrane functions as a selective osmotic barrier, while the outer wall maintains the structural rigidity of the cell. The bacterial cell is remarkably resistant to violent changes in its environment and will remain intact in media ranging from distilled water to strong salt solutions, although this tolerance is considerably reduced in the young, actively growing phase. Bacteria react by swelling or shrinking in media of high or low osmotic pressures, but the reaction is selective in that, as Fischer showed more than 50 years ago, plasmolysis of Gram-negative bacteria occurs in high concentrations of salt or sucrose but not in similar concentrations of glycerol or urea. During plasmolysis the cell wall does not shrink with the protoplast, the contents of which stream out in a series of fine droplets. Gram-positive bacteria are much more resistant to plasmolysis, but they, too, respond by contraction selectively to hypertonic solutes of sucrose but not of glycerol. This indicates that they resemble Gram-negative bacteria except that the plasma membrane does not easily separate from the cell wall, although there is good evidence that it is a distinct structure. The osmotic pressure inside the cell may be as high as 20 atmospheres, and the tensile strength of the cell wall, greater in spherical than in cylindrical organisms, is responsible for withstanding the high pressures. Obviously if this tensile strength is weakened, as by the action of penicillin, the phenomenon of plasmolysis or plasmoptosis will readily occur.

The functionally active plasma membrane is composed of lipid and protein and is estimated to be 15 mμ thick if hydrated, or 5 mμ if unhydrated. This corresponds to the classical semi-permeable membrane 2–4 molecules thick. The cell wall must also be permeable, and Mitchell and Moyle (1956) suggested that it consists of a network of chains or fibres, with an effective pore diameter of about 1 mμ. Indeed, although the plasma membrane is the important osmotic barrier to small molecules, the cell wall probably acts as the barrier to solutes of 10,000 molecular weight or over. However, the cytoplasmic membrane is, according to the experiments by Mitchell and Moyle and others, more than a semi-permeable barrier, for it contains the bulk of the cytochrome system and other enzymes, and it may act as a carrier of substances into and out of the cell, a process called exchange diffusion. This may explain the selectivity of the osmotic barrier, which would on this hypothesis be dependent on the presence of carrier enzymes which act as ferry boats for the transport of selected passengers into and out of the cell.

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THE CELL IN CULTURE

BY

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It is now more than 50 years since Ross Harrison (1907) inaugurated the technique of tissue culture *in vitro*, and at the same time settled for ever the controversy as to the origin of nerve fibres; he explanted fragments of a tadpole's spinal cord in a drop of lymph spread on a coverslip sealed to a hollow-ground slide and observed the outgrowth of naked nerve fibres into the surrounding fluid. During the next few years the work of Carrel and Burrows enabled many other cell types to be grown in culture for long periods; since then tissue culture has rapidly developed until now it ramifies far and wide into cell biology, cancer research, physiology, biochemistry, pharmacology, virology, and many other branches of science. To-day I am going to talk about the contribution that the technique has made to our knowledge of the structure and behaviour of individual living cells, as this aspect of the subject seemed the most appropriate to the present occasion.

The hanging-drop method used by Ross Harrison in his classical experiments on nerve outgrowth remains the standard technique for studying individual cells in culture. There are many different forms of hanging-drop preparation, but typically it consists of a coverslip sealed over a hollow-ground slide; the tissue fragment or explant is contained in a drop of nutritive medium, usually a mixture of plasma and embryo extract, spread on the under surface of the coverslip. When the culture is incubated at body temperature, amoeboid cells crawl out of the explant which soon becomes surrounded by a broad zone of new growth in which mitotic division is active. Every few days the tissue has to be cut out of the clot and transferred to a fresh medium or the cells degenerate.

Methods have been devised to avoid this periodic disturbance of the tissue. One is Maximow's double coverslip technique to which I shall refer later. Another is some form of continuous perfusion of the cells with an oxygenated fluid nutrient; there are many types of perfusion chamber, but in our laboratory we use the Buchsbaum apparatus (Buchsbaum and Kuntz, 1954) in which cells can be observed with phase contrast under the highest magnifications and remain in good condition for many days.

During the early period of tissue culture only two optical methods were available for the study of living cultures: ordinary direct illumination and the dark-ground microscope. Direct illumination was the more commonly used, and with its aid many accurate and beautiful observations were made by such pioneers of tissue culture cytology as Margaret and Warren Lewis in the United States, Giuseppe Levi in Italy, and by my old chief, Dr. T. S. P. Strangeways, in this country.

Let us consider how tissue culture cells appear when viewed on the warm stage of the microscope with direct lighting. In his book published in 1924, Dr. Strangeways describes the emergence of a fibroblast from an explant of the choroid and sclerotic of a chick embryo in the following words:

> "A slender process of cytoplasm is seen to protrude from the edge of the explant; this process enlarges, the nucleus passes into it, and gradually the whole mass of cytoplasm detaches itself and passes into the medium. . . . The cytoplasm of the outwandering cell appears as a clear homogeneous, jelly-like substance with no obvious cell wall, although the limits can be readily discerned. . . . The outline is constantly changing, and larger or smaller processes are thrown out and withdrawn in amoeboid movement."

He mentions rod-like mitochondria, fine granules, and fat droplets moving in the cytoplasm. There is little to be said about the nucleus of which he writes:

> "The definite nuclear wall usually shown in fixed preparations is not seen in cells growing *in vitro*. The nucleus contains one or more irregular nucleoli, which are constantly altering in shape and size."

Nothing more can be seen in the nucleus by direct illumination.

Dark-ground illumination (Strangeways and Canti, 1926, 1927) gave a much better picture of the cytoplasm than ordinary direct lighting (Fig. 1). In a living fibroblast, bright, worm-like mitochondria could be seen wandering about the cell, constantly changing shape and sometimes break-

FIG. 1.—Embryonic chick fibroblast in culture, as seen by dark-field illumination. In the cytoplasm the filamentous mitochondria and fat globules are very distinct, but the nucleus appears "optically empty" apart from the two nucleoli. (Drawing by H. B. Fell; from T. S. P. Strangeways and R. G. Canti.)

ing in two; also conspicuous were the intensely refractile lipid globules and rather less bright metabolic granules. The nucleus, however, appeared optically empty, apart from the nucleoli. The dark-field method had the disadvantage that the strong light killed the cells after about 30 minutes' exposure.

The whole course of mitotic division can be followed readily by direct illumination, but not in the dark field, where the chromosomes are difficult to distinguish and are usually obscured by the refractile material in the cytoplasm of the rounded mitotic cell. I well remember my delight when, as a young research student on a visit to Dr. Strangeways' laboratory in 1922, I saw a dividing cell in culture for the first time. My previous acquaintance with the mitotic process had been through the medium of a series of black and white diagrams which I had regarded with a certain scepticism. As I watched the living cell pass through division, I saw, rather to my surprise, that after all my textbook pictures were quite correct; yet how utterly different was the impression made on my mind by the living, moving cell in the throes of reproduction from that conveyed by the diagrams!

During the first 20 years of tissue culture, living cells were studied entirely by continuous observation on a warm stage, and very arduous work it could be. There is a celebrated entry in Dr. Strangeways' notebook which reads, "Cell watched for 49 hr.—did not divide." At the time he was investigating the intermitotic interval in chick fibroblasts; sometimes they failed to undergo a second division, which was very frustrating to the observer. However, many of these vigils were rewarded with success, and Strangeways (1922) eventually recorded the mitotic interval for chick fibroblasts as between 11 and 12 hours.

Towards the end of the 'twenties, attempts were made to record the behaviour of living cells in culture in a less laborious way, by means of cinematography. A triumphant success was achieved in this field by a London clinical pathologist, Dr. Ronald Canti of St. Bartholomew's Hospital (Canti, 1928, 1929), and I shall never forget the sensation that his film of cell migration and mitosis created when he showed it at the first Congress of Cell Biology at Budapest in 1927.

Many fascinating films of living cells followed which, when subjected to careful frame-by-frame analysis, have provided much valuable information about cell behaviour. Thus, in 1931, W. H. Lewis made a ciné-record of a feature of cytoplasmic physiology, the importance of which has only recently begun to be appreciated. This was the phenomenon that he termed "pinocytosis" or "drinking by cells."

> "Certain cells in our cultures," he writes, "especially the macrophages, take in globules of fluid from the surrounding medium. . . . By pinocytosis the cells are able to take in substances which cannot diffuse into them or be taken in by ordinary phagocytosis of semi-solid particles."

Pinocytosis has now been recorded in a wide range of cell types in culture. It has even been seen and filmed in nerve fibres, the amoeboid ends of which ingest fluid droplets which then stream up the fibre towards the cell body (Hughes, 1953). Thus speculations about the permeability of the cell membrane become rather futile when the cell can engulf globules of the fluid in which it is bathed.

Many fine films of normal and malignant cells in culture have now been made, among which may be mentioned those of Dr. Gey and Dr. Charles Pomerat in the United States and of Professor Pulvertaft in this country.

The next major advance in the study of cells in culture was the advent of phase-contrast microscopy shortly after the end of the second world war. My former colleague, Dr. Arthur Hughes,

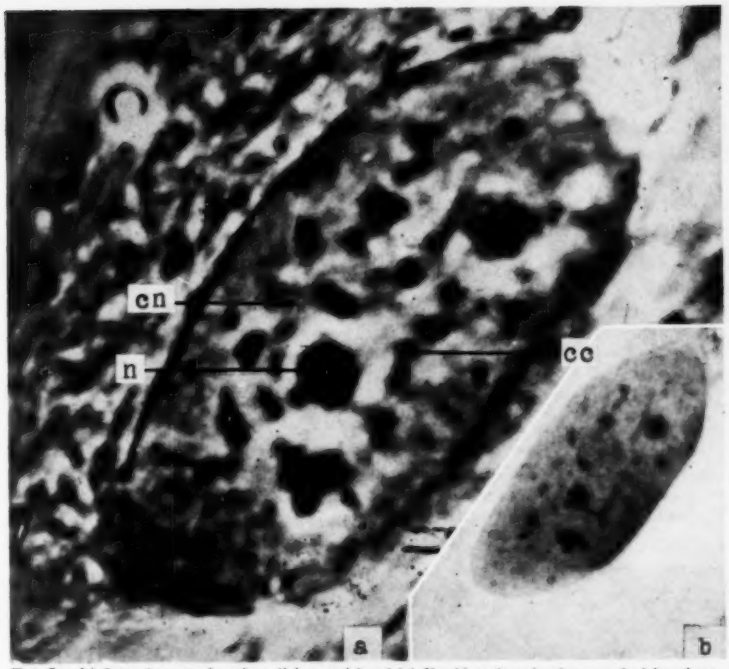

FIG. 2.—(a) Interphase nucleus in a living perichondrial fibroblast (newt), photographed by phase contrast. The chromosomal threads or chromonemata (cn), nucleoli (n), and chromocentres (cc) are shown. × 3,500. (b) Same nucleus after fixation and Feulgen-staining. × 1,350. (J. Boss.)

was one of the first to apply phase contrast combined with cinematography to tissue culture (Hughes and Swann, 1948); he made his own phase plates and cinema apparatus, and obtained surprisingly good results with his simple equipment. Nowadays the big optical firms have developed phase-contrast microscopy to a high level of efficiency, and it has become the standard method for studying living cells *in vitro*.

The most striking difference between the image of the cell viewed by direct or dark-field illumination and that seen by phase contrast is in the nucleus. As I have already said, by the older methods the nucleus appears optically empty except for the nucleoli, but with phase contrast other structures are seen. Dr. Hughes and I (Fell and Hughes, 1949) investigated the interphase nuclei of mouse spleen fibroblasts in culture, and found that we could distinguish the chromosomal threads or chromonemata as fine filaments emerging from a number of refractile granules of different sizes: the chromocentres, some of which were stuck to the inner surface of the nuclear membrane and others to the nucleoli; in fixed and stained preparations the chromocentres are Feulgen-positive. My former colleague, Dr. Jeffrey Boss (1954), found that these nuclear structures are even more obvious in the large cells of the newt (Fig. 2).

Mitosis is extremely clear in cells viewed with phase contrast (Fig. 3) and recently has been studied extensively by Dr. Boss in newt tissue cultures (1954). In this very favourable material he has been able to follow in great detail the transformation of the daughter chromosomes at anaphase into the fine chromonemata of the interphase nucleus (Fig. 4), a nice example of the structural continuity of the chromosomes postulated by geneticists.

In the cytoplasm the mitochondria and cell granules are as distinct with phase-contrast as with dark-ground microscopy. Fine cinematographic studies of living mitochondria have been made by Chèvremont and Frederic (1952) and have added greatly to our knowledge of these structures.

Of recent years the tissue culture cytologist has been provided with two more weapons: the ultraviolet and the interference microscopes. With the former, the synthesis of nucleotides can be studied quantitatively in the living nucleus, and with the

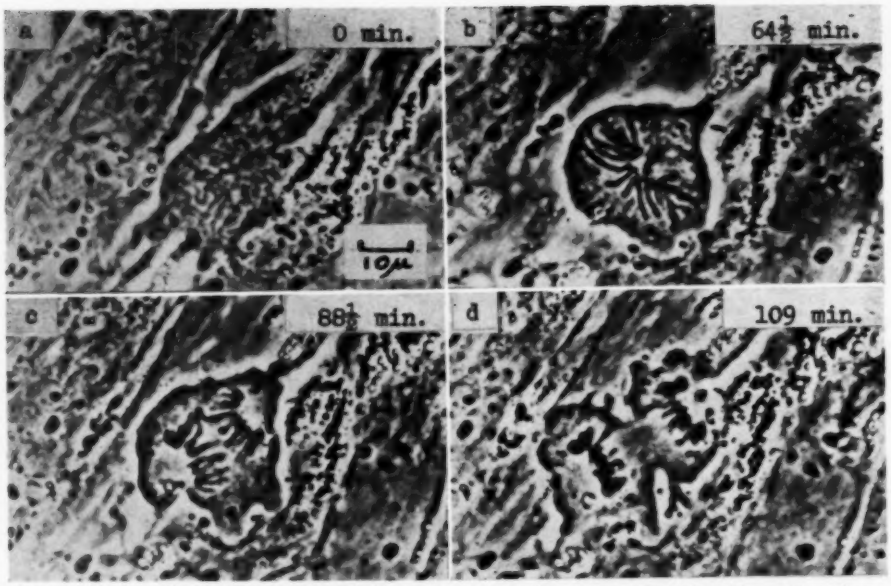

FIG. 3.—Living heart fibroblast (newt) photographed by phase contrast at different stages of mitotic division. (a) prophase; (b) metaphase; (c) anaphase; (d) telophase. × 800. (J. Boss.)

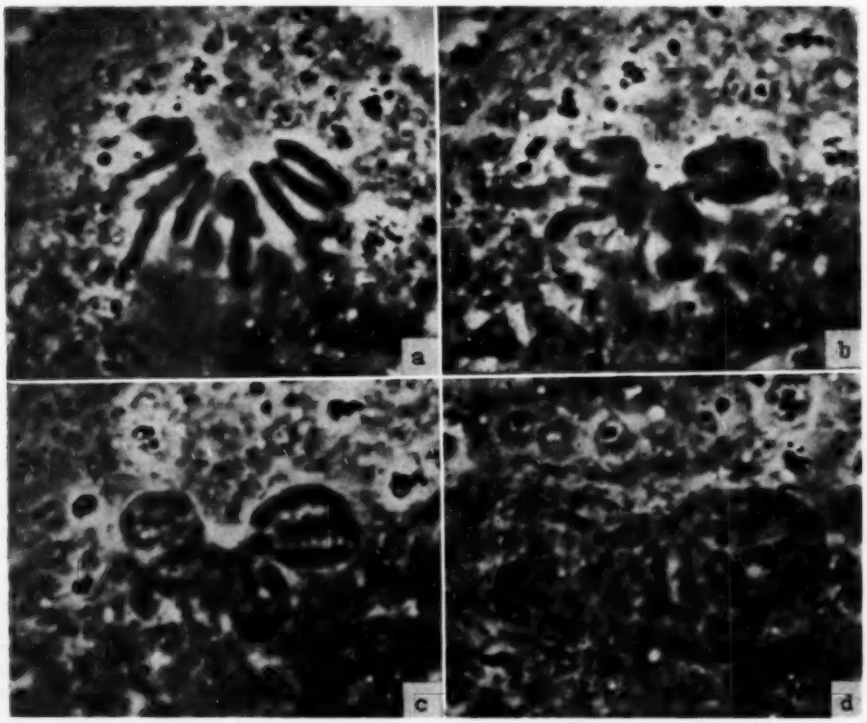

FIG. 4.—Stages in the formation of an interphase nucleus in a living perichondrial fibroblast (newt). (a) anaphase: a group of discrete daughter chromosomes are seen. (b) 12½ min. later: the chromosomes are clumped together. (c) telophase, 20 min. after (a); chromosomes again discrete; note transverse interchromosomal bridges. (d) 30 min. after (a): daughter nucleus formed. × 1,500. (J. Boss.)

latter changes in dry mass in different parts of the cell can be observed.

It is possible to investigate not only undifferentiated but also differentiated cells in culture. For example, I myself (Fell and Robison, 1930) have watched osteoblasts forming bone in cultures of osteogenic mesoderm from the mandible of a chick embryo. First, very thin refractile partitions appear between the osteoblasts, then the osteogenic fibres develop and increase in number to form an intricate network around the cells. Quite suddenly the identity of the fibres becomes indistinguishable, sometimes overnight, and the osteoid matrix appears homogeneous. Soon afterwards calcification begins as a deposit of brilliantly refractile granules throughout the intercellular material.

Very interesting work on individual differentiated cells has been done by Dr. Margaret Murray and her colleagues in New York. They use Maximow's double-coverslip technique. By this method the explant and culture medium are placed on a small round coverslip which is stuck to a larger coverslip with a small drop of saline; the big coverglass is then inverted over a large hollow-ground slide to which it is hermetically sealed in the usual way. Every few days the preparation is opened, and the little coverslip is detached and washed in physiological saline; a small quantity of fresh medium is added to the explant, and the small coverslip is placed on a clean large one which is again sealed over a hollow-ground slide. In this way cells can be maintained undisturbed for many weeks and often differentiate very well.

Murray and Stout (1954) have used this technique for the classification and diagnosis of certain human tumours, especially those of the nervous system, because they find that "intrinsic tissue characteristics may be emphasized by the changed environment of the culture, where the migrating cells are freed from the compression to which they are subject *in vivo* and where the cell can always be seen in its entirety."

Peterson (1950) kept embryonic ganglion cells in culture for many weeks, and during that time the long nerve fibres formed by the cells acquired their myelin sheath, a process that could be

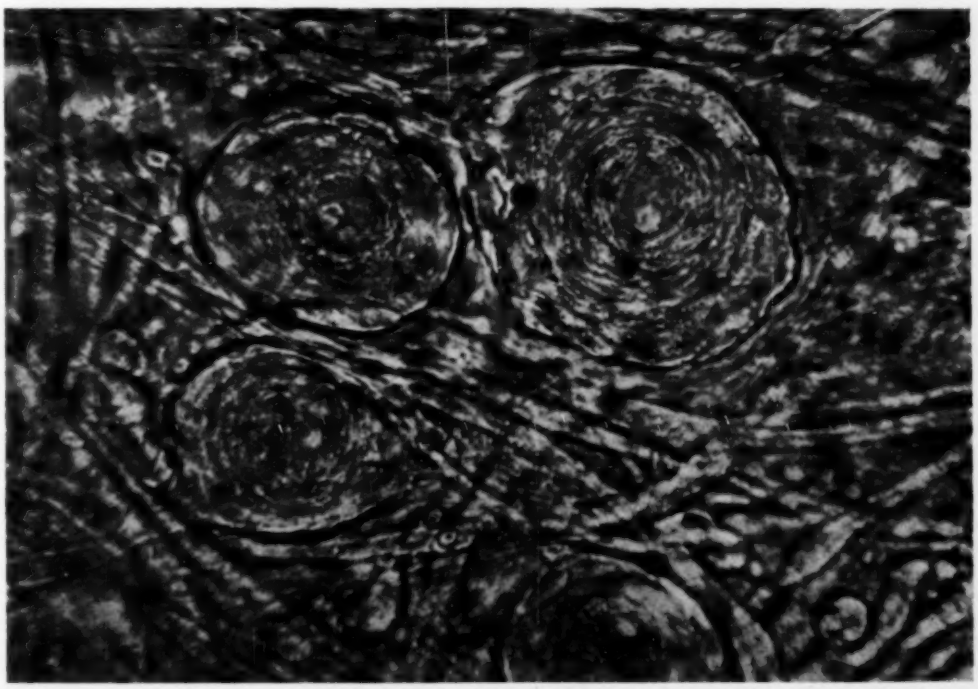

FIG. 5.—Living neurons of a dorsal root ganglion from a nine-day chick embryo; 20 days in culture. The neurons develop as large flattened discs with satellite cells and Schwann cells firmly pressed against neuronal bodies and axones. × 900. (E. Peterson.)

FIG. 6.—Differentiating mononuclear cell in a 30-day culture of adult human leg muscle. Fibrillae and irregular cross-banding are seen. × 900. (I. A. Pogogeff and M. R. Murray.

studied in detail in the living tissue. When I visited Dr. Murray's laboratory recently, I saw one of these cultures. It was a fascinating spectacle with its large round nerve cells, in which the nucleus and Nissl substance (Deitch and Murray, 1956) could be clearly distinguished (Fig. 5), and long refractile myelinated fibres winding their way through the tissue. Pogogeff and Murray (1956) have grown adult human skeletal muscle for several weeks and obtained cross-striated contractile muscle fibres (Fig. 6).

The fact that individual living cells can be observed under the highest magnification not only enables normal cellular processes to be observed in life, but makes it possible to investigate the cytological effects of many experimental agents such as drugs and radiations, and also to watch the intracellular behaviour of certain viruses (cf. Bland and Canti, 1935).

In this short talk I have merely selected a few illustrative examples of the cytological aspects of tissue culture, because a review of the large literature would have been out of the question. During the past 36 years I have been able to watch the development of the subject at close quarters, and I have tried to convey to you something of the story that has unfolded before me during that time.

I am deeply indebted to Dr. J. Boss, Dr. Margaret Murray, and Dr. Edith Peterson for permission to reproduce photographs and for kindly supplying prints. I wish to thank the editors of the *Quarterly Journal of Microscopical Science* and the Academic Press (publishers of *Experimental Cell Research*) for allowing me to reproduce Figs. 1 and 2 respectively.

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CYTOCHONDRIAL ASPECTS OF CELLULAR PATHOLOGY

BY

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Cytochondrial Composition of Cytoplasm

Although the introduction by Purkinje (1825) of the term protoplasm may have established a conceptual advance, it also provided for over-simplification and led to the separation of cytoplasm into hyaloplasm and granuloplasm, with emphasis on the former. This dichotomy is still apparent in the emphatic works of numerous biologists (Seifriz, 1942; Frey-Wyssling, 1948), who relegate the granular constituents of cytoplasm to incidental roles. Accumulated evidence has reversed this trend and redistributed the cytoplasmic structure into multiple particulates. The Pasteur and Crabtree effects with particulate and soluble systems from the cytoplasm have indicated that the phenomena are mediated by interaction between these components (Aisenberg and Potter, 1957). Current perspective accords to cell particulates an essential participation in living processes, so that it is imperative to evaluate their significance and importance in cellular pathology.

By utilizing such diverse techniques as phase-contrast microscopy (Zollinger, 1950), electron microscopy (Dalton, 1953), and the homogenization of cells with controlled differential centrifugation (de Duve and Berthet, 1954), a basic concept of particulate cytoplasmic structure has been evolved. The range of particles extends from the easily visible mitochondria and the heterogeneous microsomes to the units appreciated only by electron microscopy. Schemes of subdivision have been advanced which differ only in detail concerning certain borderline particles (Novikoff, 1956a; Kuff, Hogeboom, and Dalton, 1956). We refer to all cytoplasmic organelles collectively as cytochondria (Opie, 1948; Kitiyakara and Harman, 1953).

One scheme of cell structure devised by Novikoff (1956a) is shown in the illustrations of the rat parenchymatous liver cell (Figs. 1–4). It has been used by cytochemists (Claude, 1946) and pathologists (Christie and Judah, 1953) for analysis of cytoplasmic reactions. It comprises a manageable system for the elucidation of pathological events at subcellular level. A recent study of uterine mucosa shows a similar picture (Nilsson, 1958). The mitochondria, which had been observed and isolated from cytoplasm in the early nineteenth century (Kölliker, 1857), were established as cytoplasmic constituents by Altmann (1894) in a study of cytoplasmic structure, using freeze-dry technique, and were isolated in bulk for chemical analysis by Bensley and Hoerr (1934) and by Hogeboom, Claude, and Hotchkiss (1946). It was observed that the integrated enzymes, which catalyse Krebs's tricarboxylic acid cycle and oxidative phosphorylation (Green, Loomis, and Auerbach, 1948), were located almost exclusively in the mitochondria (Harman, 1950a). It was proposed that mitochondrial integrity and function of the integrate cycle were mutually dependent (Harman, 1950b; Judah and Williams-Ashman, 1951). Although the contention that the entire Krebs's cycle resided in the mitochondria was challenged (Schneider and Hogeboom, 1951) a great deal of evidence identified the component enzymes within mitochondria. With the recent observations of Ernster and Navazio (1957) it can be accepted that the cycle can operate exclusively within these units. The importance of this fact to cell metabolism and pathology is stressed by the quantitative predominance of the particles, which comprise 30% of cell mass and number 2,500 per cell tightly packed (Allard, Mathieu, De Lamirande, and Cantero, 1952), as illustrated in Fig. 5, which is a photomicrograph of a rat liver cell prepared by Kaltenbach's method (1954). The characteristic fine structure of the mitochondria has been catalogued for many tissues by Palade (1952), and this structure, with inner lamina and outer interface, is shown in Fig. 6. It has been disputed in several types of mitochondria (Weinreb and Harman, 1955; Powers, Ehret, and Roth, 1955), as shown in Fig. 7.

The significance of this ultrastructure has been strengthened by the correlation of various enzymes and systems with its constituents. The

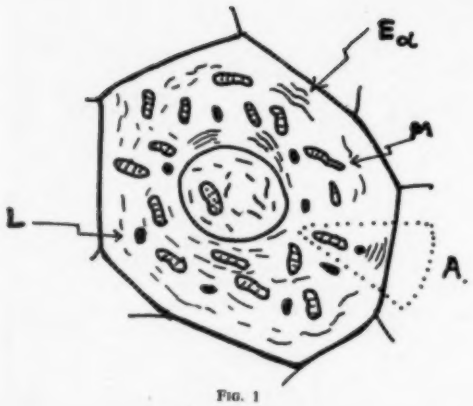

FIG. 1

FIG. 2

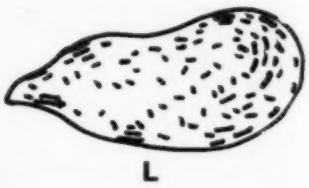

FIG. 3

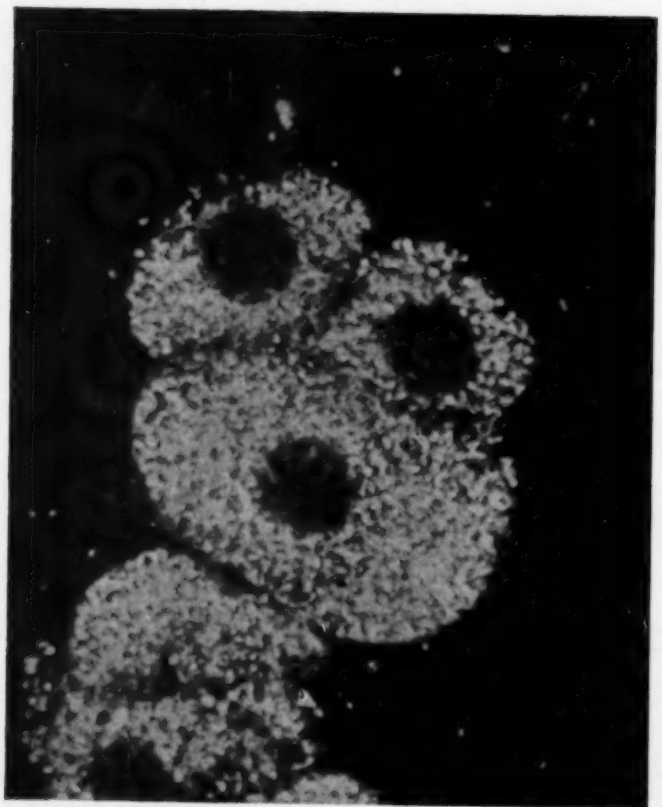

FIG. 5.—Several liver cells isolated in a metabolically active state are illustrated with light contrast phase microscopy. The cytoplasm is completely crammed with dense, rodlet mitochondria. Cells isolated by Kaltenbach's technique (1954) in buffered saline. × 1,500.

FIG. 1.—Diagrammatic representation of parenchymal cell particulates includes (M) mitochondria, (L) lysosomes and fat bodies, and (E) orgastoplasm or endoplasmic reticulum. Segment A components are magnified in Figs. 2, 3, and 4.

FIG. 2.—The mitochondrion is depicted, with looping, doubled membranes and lamellae. At "a" a peripheral loop is indicated, and at "b" the double membrane is replaced by a single interface. This was drawn from representative liver mitochondria. The inner fine gel contains several highly electron-dense particles, which are especially large in uterine mucosal cells.

FIG. 3.—The lysosome is drawn after the representations of Novikoff *et al.* (1956). A characteristic interior particulate composition is evident from his electron micrographs, and the fibrous gel of the mitochondria is conspicuously absent.

FIG. 4.—Two interpretations of the endoplasmic reticulum are presented. In S the observation of Sjöstrand, which regards the network as ribbon-like, is manifest, and the inner granules adherent to the lining of the interface. In P, which displays Palade's view, the orgastoplasm is regarded as a series of concatenated vesicles, each with its complement of granules. The granules are interpreted as ribonucleic acid particles.

FIG. 6.—Portion of a rodlet mitochondrion in a kidney cell manifests the inner laminae and surface loops, which appear to fuse as limiting surface membranes. This is an illustration from Rhodin (1954) by permission. Many electron opaque inner particles are visible.

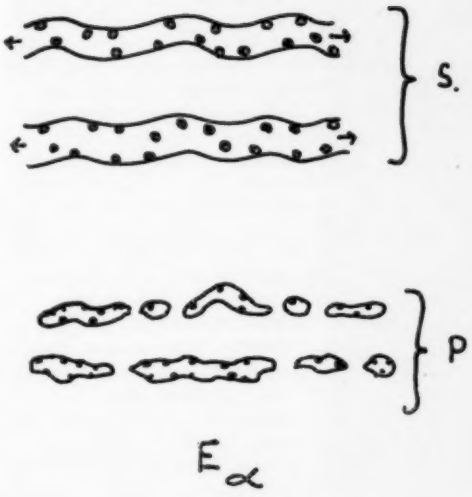

FIG. 4

FIG. 6

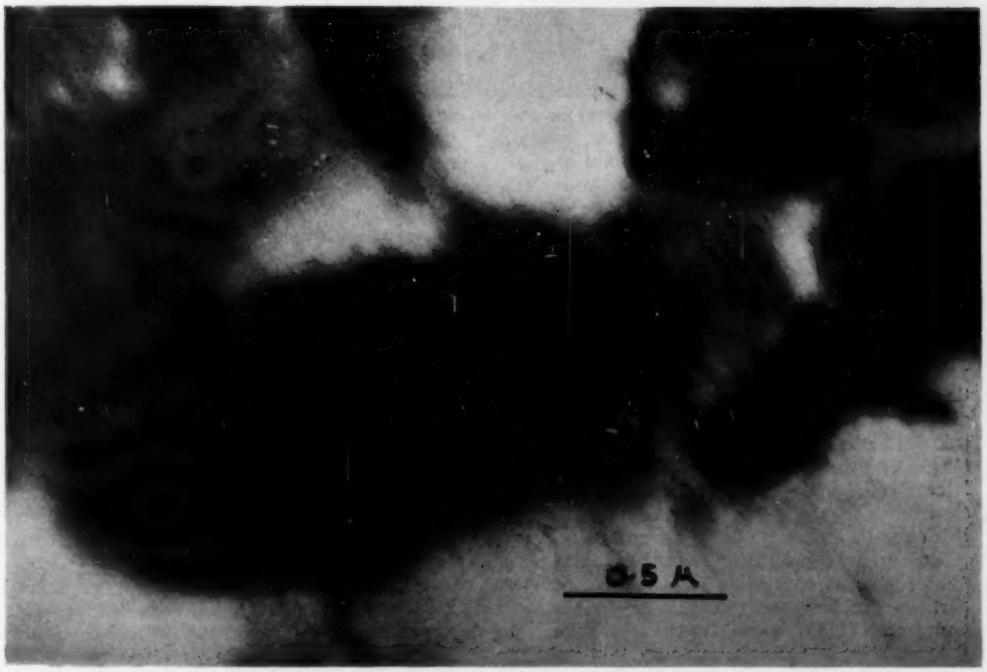

FIG. 7.—A mitochondrion from skeletal muscle of pigeon breast is composed of transverse laminae, without limiting peripheral double membrane. Electron drift was excluded in the photomicrograph by clear demonstration of the perpendicular fibrillae in the myofibrils, indicating that lack of surface membrane is not artifactual.

adenotriphosphatases of the mitochondrion are bound to the ribbed hull and defy elution, although a considerable portion of the nitrogen may be made soluble (Harman and Kitiyakara, 1955). On the other hand, the succinoxidase system and the electron chain occupy a sequential distribution in the laminae, and are obtained by mechanical, sonic, and digitonin disruption, which yields respiratory assemblies capable of phosphorylation (Ziegler, Linnane, and Green, 1958; Crane, 1958; Kielley and Bronk, 1958; Cooper and Lehninger, 1956). Furthermore the susceptibility of the mitochondrial matrix and folded inner fibrous gel to swell and change their form depends on the redox state of electron carrier assemblies on the laminae (Hunter, Davis, and Carlat, 1956). When carriers are reduced there is limited reactivity to agents such as thyroxin (Lehninger and Ray, 1957). It is apparent therefore that the nutritive condition of the cell determines its reaction to injury.

There are particles of the same dimension and of similar pyknic gravitational density with mitochondria, one of which has been designated "lysosome" by de Duve, Pressman, Gianetto, Wattiaux, and Appelmans (1955) and further identified by electron microscopy (Novikoff, Beaufay, and de Duve, 1956). Its separation from mitochondria by differential gradients indicates that this unit is peculiar because of an exceptional content of hydrolytic, phosphoroclastic enzymes and glucuronidase. The injurious consequences on cell activity when their enzymes are released is apparent. The second particle, located in liver, is associated with iron metabolism and so has been called "siderosome" (Richter, 1957). Some very minute mitochondria, such as the "ultramitochondrioma" of Oberling, Bernhard, Febvre, and Harel (1951), have received special treatment and interpretation as regenerative precursors.

Microsomes (Claude, 1946) were for many years a heterogeneous group. They were distinguishable from heavier particles not only by difference in size and density but by a characteristically high content of ribonucleic acid. Monné (1948) discussed them as the chromidia of the intact cell. With better understanding from electron microscopy (Palade and Siekevitz, 1956) it is clear that they are derived from endoplasmic reticulum or ergastoplasm which is a series of minute vesicles containing tiny ribonucleoprotein particles (see, however, recent studies by Sjöstrand and Baker (1958) which contest the validity of Palade's (1955a) 150 Å particles of ribonucleic acid in the microsomes). The minute vesicles are arranged with some regularity in strings throughout the cytoplasm in three-dimensional dispersions and form a network which may help to maintain cell rigidity. These particles are divided into smooth- and rough-surfaced variants and are associated with lipid and protein synthesis respectively (Palade, 1955b).

These cytochondria, including the Golgi apparatus, are now the scene for current investigations in cellular pathology. It is possible to describe the mechanisms of disease in terms of disordered particles and observe how alterations of particles in some instances participate in pathogenesis.

Mitochondria in Disease

The immediate concern with mitochondria in disease is the correlation of their morphological alterations with functional disturbances. The typical rodlet configuration of normal intracellular mitochondria has been determined by cinematography with phase microscopy (Harman, 1954; Frederic, 1958) and reaffirmed by electron microscopy for many tissues (Palade, 1952). Numerous investigators have established *in vitro* and *in vivo* that departure from this shape, by enspherulation or rarefaction, is a response to various stress agents (Harman and Feigelson, 1952; Cleland and Slater, 1953). More recently a comprehensive review of these investigations using electron microscopy confirmed the observations determined at the light level (Sjöstrand, 1956; Rhodin, 1954). The phenomena occurring within the cell in response to stress have been grouped as the process called "cloudy swelling," in which the most salient derangement is the distortion of mitochondrial morphology.

The fundamental participation of the mitochondria in the process of cloudy swelling was established by Anitschow and Aschoff in 1914, thus extending the work of Fauré-Fremiet (1910). Imposition of hyposmolarity, toxaemia, starvation, and ischaemia uniformly effect the same mitochondrial alteration and cloudy swelling. Later workers repeated the pioneer experiments and confirmed again the association of mitochondrial enspherulation and rarefaction with cloudy swelling (Zollinger, 1948). Fig. 8 is a replica of the cells depicted in Fig. 5 after the application of hyposmolar stress and transformation. Recently Opie (1948) demonstrated that not only does an increase of mitochondrial volume characterize cloudy swelling under hyposmosis but basophilic material disappears from the cytoplasm, presumably out of the ribonucleic acid particles. This

Drochmans (1947) confirmed by chemical analysis. Zollinger (1950) reviews all the evidence for the major role of the mitochondria in cloudy swelling.

A major advance in cytological technique occurred when media were improved allowing morphologically intact mitochondria to be isolated from the cell (Dounce, Witter, Monty, Pate, and Cottone, 1955; Novikoff, 1956b; Birbeck and Reid, 1956). The steady refinement of isolation methods has provided several media which satisfy most of the ultrastructural criteria of normal segregated particles. Moderate hyperosmolarity with selected non-electrolytes, careful *p*H adjustment, refrigeration, and elimination of noxious contaminating ions by chelation enable native fresh mitochondria to be obtained. Considerable analysis has emphasized the deleterious effects on mitochondrial morphology (Harman, 1956) of any departure from these restrictions. With native mitochondria so obtained it has been possible to elicit the phenomena of cloudy swelling in the free, uncontaminated units.

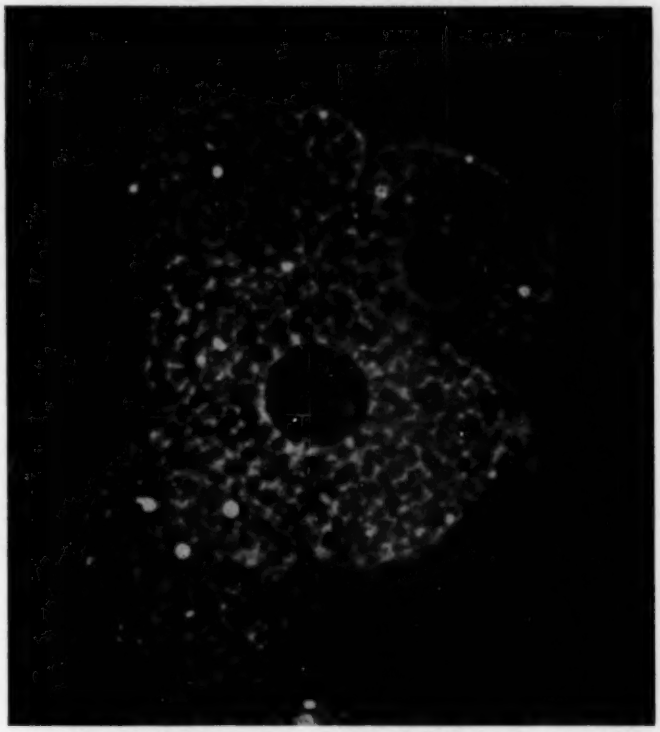

FIG. 8.—The identical cells illustrated in Fig. 5 are seen after brief exposure to deionized water at 25°. Slight swelling is obvious, with vesiculation and blurring of the inner structure. Contrast seems reversed although light contrast conditions still apply. × 1,500.

The mitochondria isolated under ideal conditions are described as "resting" particles. So long as they remain in rodlet form, their metabolism and phosphate exchange is minimal. After variable periods of "ageing," even at low temperature, changes in form begin, and the metabolism of the integrated enzyme systems are more ungovernable when they are regarded as "aged" preparations, with the connotation of a specific morphological and metabolic deformity. The process of "ageing" may be accelerated by applying temperature gradients, osmolar variation, minute quantities of certain ions, e.g., Ca^{++}, at levels below osmolar influence, and by mechanical agitation as well as by certain organic solvents. These are the various types of transforming agent and their overall effect is seen in the occurrence of mitochondrial swelling and the formation of distinctive spheres with crescent-shaped marginal condensations. Fig. 9 shows mitochondria from skeletal muscle suspended in deionized water. Several reports have confirmed the sequence of events which lead to swelling and have established the alterations of hydration, dry weight, and optical density accompanying the change. Ionic radiation has a similar effect (Fig. 9a).

The morphological alterations distinguished above under the impact of transforming agents are attended by profound disturbance in the mitochondrial metabolic activities. It has been well confirmed that the Krebs's cycle operates as an integrated system within the mitochondria, and is facilitated by the intimately associated Keilin-Hartree electron chain which is confined to the mitochondria. It is thus that the sequential oxidation of the moieties of the cycle are concatenated with the traversing of the electron chains and the development of high energy phosphates in the nucleotide pools which the mitochondria contain (Figs. 10, 10a). This high energy, especially as adenosine triphosphate

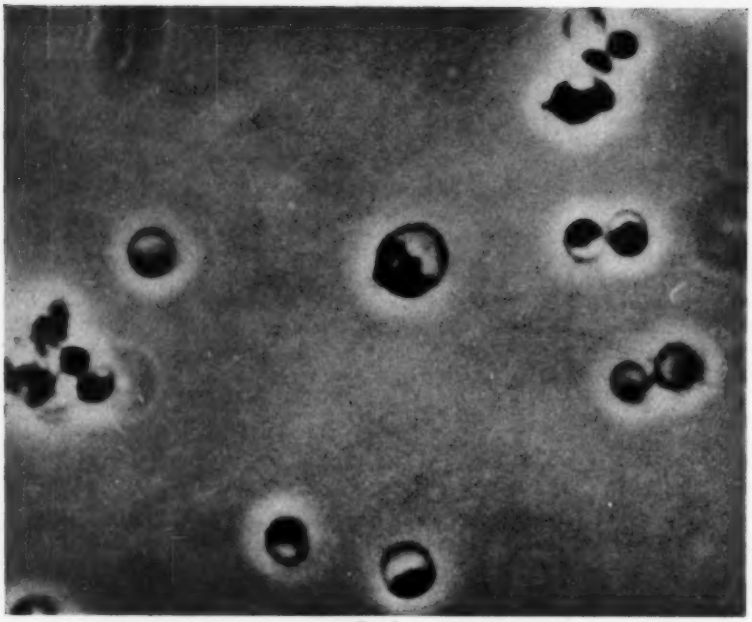

FIG. 9

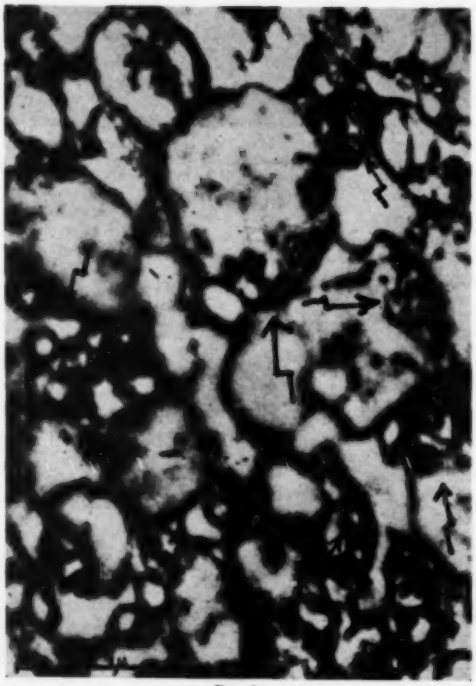

FIG. 9a

FIG. 9.—Mitochondria isolated from skeletal muscle by the method of Kitiyakara and Harman (1953) are preserved by osmium fixation after exposure to deionized water. Typical crescent transformation, which was seen in the whole cell of Fig. 1, is the dominant alteration. Phase contrast, dark L conditions used. × 2,900.

FIG. 9a.—A suspension of mitochondria which were exposed to ionizing radiation, with transformation into typical crescents. Arrows indicate the lamellae concentrated along a margin of the bloated particles. × 22,000 (from Okada and Peachey, 1957).

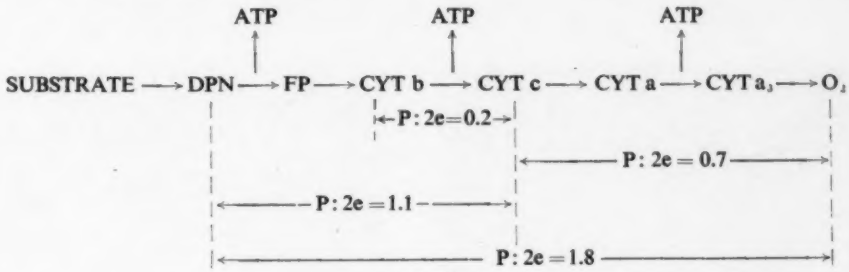

FIG. 10

FIG. 10.—This is illustrative of the established sequence of the Keilin-Hartree electron chain, with the points where high energy is tapped off into ATP traps, which are confined within cross-over sites.

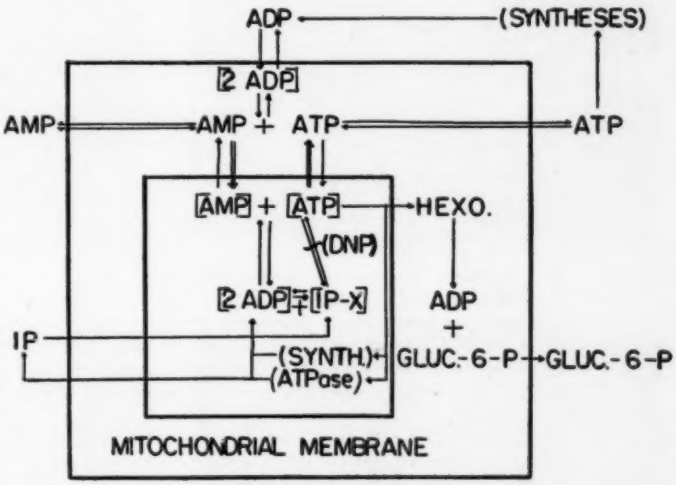

FIG. 10a

FIG. 10a.—The nucleotide pool within the mitochondria is essentially a system employing ADP as acceptor for high-energy phosphate, with some myokinase participation. Sites of action of ATPase, DNP, and synthesis are suggested.

(although it may take other forms), is available in the first instance for the maintenance of mitochondrial integrity itself and also for the provision of energy to maintain other structures and functions of the cell. Ball and Barrnett (1957) have allocated the disposition of separated oxidase systems from the cycle to the inner membrane fractions derived from isolated mitochondria, and Devlin and Lehninger (1956) have obtained phosphorylation with such fractions. Hatefi and Lester (1958) have detached heavy and light lipid components from the heart mitochondria and detected organization of an enzyme sequence which permits phosphorylation by the separated oxidases, e.g., the alphaketoglutaric oxidase group.

The distortion of mitochondrial morphology has therefore been associated primarily with a disruption of the organized sequence of the Krebs's cycle and with the augmentation of unrestricted oxidation of metabolites. This chaotic state is not attended by phosphorylation, so that the process of transfer of energy into the nucleotide reservoirs is impaired and the energy level of the units is depleted. The decrease in available high energy adenosine triphosphate permits further swelling and dissolution of the units into hulls which are beyond reconstruction. Both Raaflaub (1953) and Ernster (1956) believe that adenosine triphosphate fits into the structure whereas Harman and Feigelson (1952) prefer to regard it as a source of energy for structural re-synthesis, as for hydrogen bonding. If this happens within the cell the processes dependent on the flow of high energy from the mitochondria are in abeyance, and if they are critical to cell economy cell death is inevitable.

The phenomenon of cloudy swelling and mitochondrial structural deformity is not a simple matter of separating oxidation and phosphorylation. The gamut of phosphoroclastic enzymes

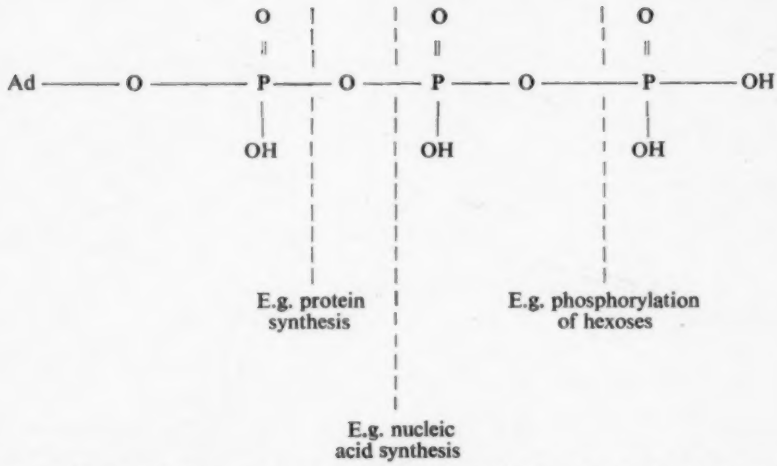

FIG. 11.—The sites of utilization of the high energy phosphate and splitting of PO bonds is shown, knowing that the same enzymes may employ H_2O as well as the specific receptors indicated, when organization is deranged (cf. Grabe, 1958).

which are galvanized into motion have an especial propensity and specificity for cleaving nucleotides concerned with electron and high energy phosphate transfer. The adenosine triphosphates, pyrophosphatases, and phosphatases operate at several levels and strike at the points concerned in the phosphorylation processes designed for the anabolic cellular functions. When disturbance occurs in normal concatenated enzyme cofactor associations and structural deformity ensues, e.g., with osmotic swelling, the enzymes capable of utilizing the P-O bonds in adenosine triphosphate for protein synthesis, nucleic acid formation, and hexose phosphorylation, among other syntheses, are labilized and split the bonds at the appointed site without transfer of energy from catabolism to anabolism (Grabe, 1958). Fig. 11 depicts the operative sites in the adenylpyrophosphate molecule for the various clastic enzymes. For example, in protein synthesis, under the agency of an activating enzyme, the pyrophosphate is sheared off and the adenosine monophosphate fragment united with the amino-acid to yield, for instance, adenylleucine. The net result of such a reversal of enzyme function is the literal piecemeal tearing apart of the cell unless some portion of the synthetic mechanism survives to compensate adequately. Since the mitochondria and lysosomes also are rich in catheptic enzymes, lysolecithin, and other lytic factors, it is remarkable that cloudy swelling is considered as reversible. To my knowledge no one has determined the survival of cells submitted to cloudy swelling, and most observations have been made on post-mortem samples. *In vitro* the state of the mitochondria equivalent to cloudy swelling is not reversible, because of gross fracture of structural units (Kaltenbach and Harman, 1955) and depletion of ribonucleic acid constituents (Fig. 12).

Action of Bacterial Toxins

Because of the especial concentration of oxidative and phosphorylating systems in mitochondria the metabolic vulnerability of the cell is often largely dependent upon them. This has provided several groups of workers with a focus for their investigations of various diseases, especially the mechanism of toxicity characteristic of certain infectious diseases. The Italian school of Fonnesu and Severi (1956) has accumulated evidence by *in vivo* studies and with mitochondria isolated from animals intoxicated with diphtheria toxin that the specific action of the active component is against the mitochondria with cloudy swelling, lowering of oxidative phosphorylation and ~ P reserve. The same effect is observed after injections of *S. typhimurium* and other bacterial extracts. Pappenheimer and Hendee (1947) have suggested that in diphtheria the toxin is the protein component of cytochrome b manufactured by *C. diphtheriae*. This is in effect an elaboration of a succinic acid dehydrogenase, which is suspected of penetrating into the mitochondrial succinoxidase system in heart muscle and kidney and of

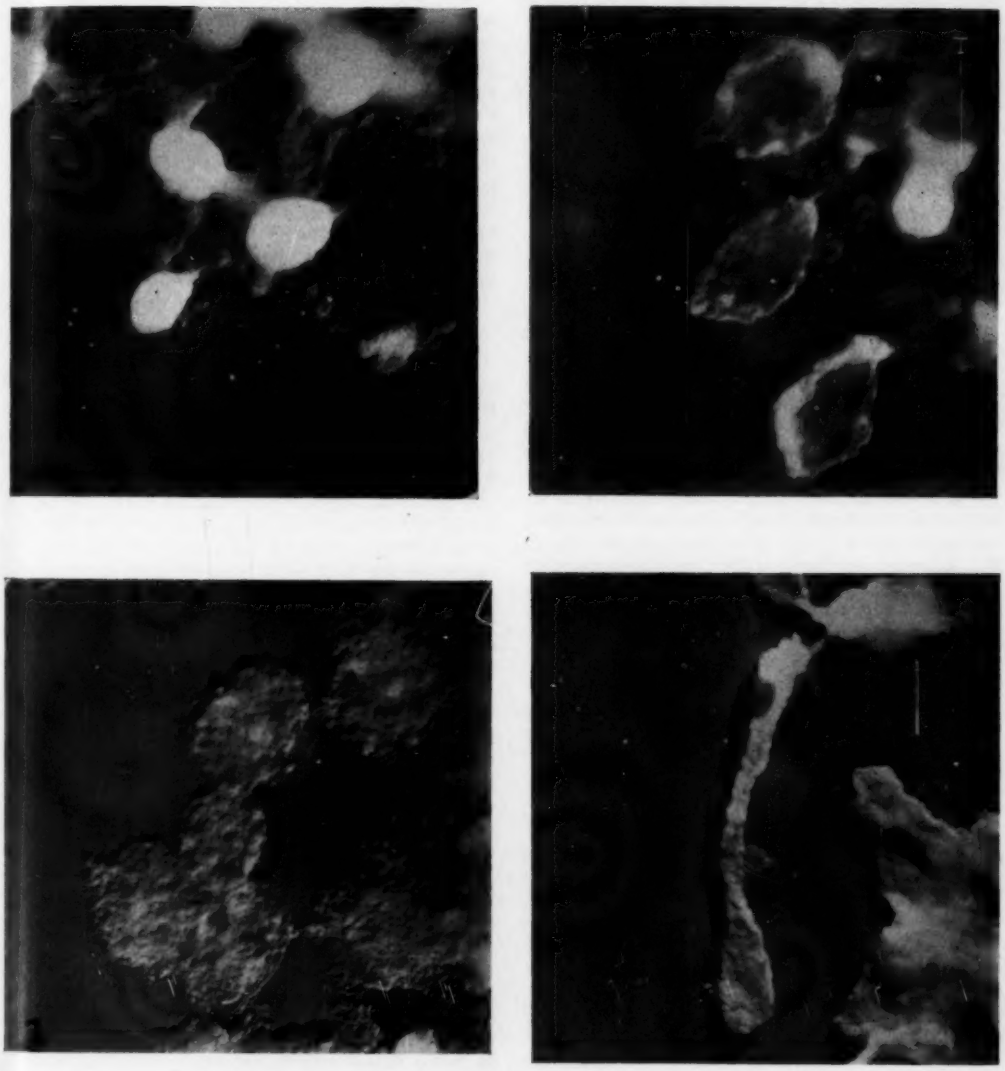

FIG. 12.—The mitochondria depicted were isolated in isosmolar sucrose which were exposed to short periods of deionized water treatment, then to thermal activation. These units then placed in hyperosmolar solution shrank, but persisted as elongated threads. (1) Original; (2) 30′ in H_2O; (3) 120′ in H_2O; (4) in isosmolar sucrose again. The original compact form was not resumed. × 11,000.

displacing the normal electron flow. Impedance of the integrated system interferes with total oxidative phosphorylation, encourages P pools to fall, and allows dispersal of mitochondrial structure or the occurrence of cloudy swelling. Although Pappenheimer and Williams (1952) have demonstrated the coincidence of the action of diphtheria toxin with the time of the appearance of cytochrome b in larvae, problems concerning the production of toxin by the bacterium are not yet clear enough to identify the toxin with the cytochrome protein. Furthermore, cytochrome c is not part of the electron path of diphosphopyridine nucleotide-dependent oxidases. Waksman, Adams, and Mansmann (1957) suggest that since the toxin interferes with phosphorylation in several types of cell, it may cause diphtheritic polyneuritis by such injury to the Schwann cells.

That several other bacterial products exert their action on mitochondria is illustrated in the following two examples. A most potent toxin from *Cl. welchii* is the alpha-toxin or lecithinase, which has the property of splitting phosphocholine from lecithin and displacing the compound from its structural position. Another enzyme, phospholipase, which occurs in snake venoms, splits the unsaturated fatty acid from lecithins and elaborates a factor called lysolecithin, which in itself has a lytic action on lipid-protein membranes. These enzymes from animal and bacterial sources have a dual action on mitochondria as the toxins directly dissolve the laminated and organized structure of the particles by destroying the lipoprotein mosaic (Nygaard, Dianzani, and Bahr, 1954) and simultaneously they hasten the swelling by inactivating a lipid containing electron transport factor located in the succinoxidase system between the cytochrome b and cytochrome c components (Nygaard, 1953). Cryptically situated lysins (Tyler, 1951) and enzymes of similar character are distributed in most animal cells and may be activated to interfere with mitochondrial permeability (Witter and Cottone, 1956). On the other hand, as a second example, certain streptococci of the β-haemolytic type produce a lysin which may profoundly affect the mitochondrial system in a different manner. Carlson, Kellner, and Bernheimer (1956) have shown that streptolysin-O from filtrates of streptococcal supernatants has the selective capacity of inhibiting the oxidative metabolism of cardiac mitochondria. Further examination of this phenomenon verifies that the inhibition is due to a streptococcal diphosphopyridine nucleotidase (Carlson, Kellner, Bernheimer, and Freeman, 1957) which destroys the co-enzyme diphosphopyridine nucleotide essential for the function of several oxidases of the mitochondria, which are therefore attacked with remarkable specificity. A similar enzyme has been detected in plague toxin (Ajl, Rust, Woebke, and Hunter, 1956), which differs slightly from the streptococcal enzyme by splitting the diphosphopyridine nucleotide at the phosphate rather than the nicotinamide-ribose junction. The importance of diphosphopyridine nucleotidase in its pathogenesis and virulence is suggested by the correlation of its activity with the leucocidal and myocardial damaging capacity of the organism (Bernheimer, Lazarides, and Wilson, 1957; Kellner, Bernheimer, Carlson, and Freeman, 1956).

Organic Toxic Compounds

Among a plethora of noxious organic chemical agents which can induce cloudy swelling, carbon tetrachloride has received exceptional attention, together with the related compound, chloroform. Several workers have employed it for the elucidation of phenomena concerned in cellular injury and use it as a prototype reaction. Christie and Judah (1953) have assessed the action as a direct structural disruption of the mitochondrial population of the cell, with disorganization of the integrated enzyme systems comprising the tricarboxylic acid cycle and an accompanying loss of co-enzymes, especially di- and triphosphopyridine nucleotides. The co-enzyme splits away at a different rate from the various oxidases, an observation which agrees with the opinion by Huennekens and Green (1950) that various enzyme systems have different tenacities for their co-enzymes. A specific block has been detected, moreover, in the system which can be explained on the basis of derangement of the normal sequence of enzyme activity. The more recent observations of Recknagel and Malamed (1958) contend that carbon tetrachloride obliterates a normal mitochondrial permeability barrier and so produces swelling, and the other consequences follow this initial change. The displacement of the co-enzyme may be the prime insult in both instances, and it would be desirable to extend the observations concerning the ketoglutarate block to determine its reversibility with appropriate co-enzyme fortification. Before accepting the thesis that the carbon tetrachloride acts directly on the mitochondrial structure it is necessary to evaluate the observations of Theirs, Reynolds, and Vallee (1958) on ion changes in mitochondria from CCl_4-intoxicated animals. There is a 16-fold rise in calcium ions immediately,

accompanied by a sharp fall in potassium. The total ion binding and content is constant. It may be, therefore, that CCl_4 destroys normal cell membrane permeability and allows calcium to sweep into the cell with its deleterious actions on mitochondrial form and function, since Ca^{++} is an established mitochondrial toxin (Ernster and Löw, 1955 ; Slater and Cleland, 1953). They also noted that simultaneously there was uncoupling of oxidative phosphorylation with all substrates of the Krebs's cycle used, although some restoration of function is possible with added diphosphopyridine nucleotide and cytochrome c. It appears likely, therefore, that the damage is similar to thio-acetamide intoxication as determined by Gallagher, Gupta, Judah, and Rees (1956).

This type of experiment leads to a consideration of the metabolic accumulations which may attend stress damage to mitochondria. It is imperative to introduce a concept at this juncture to clarify certain lesions in the cell ultrastructure which are at the junctional site of molecular structure and function. It may be feasible to group together all those lesions which lie at the border of fine structure and metabolism and are characterized by the accumulation of the various metabolites along the pathways of the organized systems. Since amassing of intermediates in unusual quantities may have injurious consequences for the system, it constitutes a special type of metabolic lesion. A good example is the lesion induced by fluoro-acetate intoxication (Liébecq and Peters, 1949) in which large amounts of citrate are dammed within the mitochondrion associated with undesirable chelation reactions. A still more naturally likely variety is demonstrated by Montgomery and Webb (1956) with parapyruvate, which is a naturally occurring dimer incidental to simple accumulation of pyruvate. It powerfully inhibits oxidative decarboxylation of oxyketoglutarate, to which it has structural similarity. It has been suggested that this similarity allows the parapyruvate to occupy the ketoglutarate binding site on the mitochondrial oxidase, and so taking part in the reaction sequence, the formation of a stable cirtramalyl derivative with co-enzyme-A or lipoic acid, and the titration out of the cofactor, so completely blocking the cycle. Examination in our laboratory of the mitochondria under the influence of parapyruvate block demonstrated a typical bizarre swelling characteristic of that found with several chelating agents and observed with cloudy swelling (Harman, 1958). It is conceivable that numerous metabolites may behave like pyruvate under the influence of genetic and toxic agents and allow injurious alternative polymerizations and racemizations to interpose metabolic blocks. The process may be called "parametabolism," without excluding its intervention to some extent in normal processes. One is thus led to consider, for example, whether the myohaemoglobinuria, peculiar to horses and some humans, which is preceded by massive glycolysis of muscle glycogen, may be caused by an accumulation of pyruvate rather than lactate and the formation of overwhelming volumes of parapyruvate which interfere with muscle metabolism so acutely as to cause structural collapse of the myofibres.

It has been conventional to follow any discussion of the phenomenon of cloudy swelling with that of fatty degeneration, thus suggesting that they are connected. This respected tendency rests on the claim that more potent noxious agents press the cell beyond cloudy swelling into fatty changes. The frequent association of the two events allows of some belief in this opinion. It is more apposite, however, to consider at this juncture whether the bloated mitochondria of cloudy swelling are converted directly to fat vacuoles or some deviation of function allows accumulation of the fat within the hyaloplasm. Some observers have separated the fat particles from fatty livers and obtained mitochondrial enzymes among them, but the unavoidable comminution of mitochondria which accompanies the homogenization technique, though small, may account for this distribution, and these workers are therefore cautious in interpreting their results. On the other hand, it is expected that since the fatty acid oxidase system is the most sensitive of all the oxidases the mitochondrial derangements are conducive to the appearance of particulate fat in the cytoplasm. The activation of fatty acid oxidation normally requires a ready source of adenosine triphosphate either from an available nucleotide pool or an actively operative mitochondrial oxidative system. Dianzani (1954) has assembled data which point to depression of the P/O ratio with substrates requiring diphosphopyridine nucleotide, such as malate, glutamate, pyruvate, and ketoglutarate, by mitochondria obtained from fatty livers developed by injection of steatogenic poisons or feeding choline-deficient diets. He expressed the opinion that the low ratios were due to the loss of phosphorylative abilities as a consequence of morphological degenerative processes. Both co-enzymes I and II and cytochrome c are considerably reduced in the fatty livers (Dianzani, 1955 ; Dianzani and Viti, 1955), and these alterations are accompanied by a sharp decrease in the adenosine triphosphate

content of the mitochondria (Dianzani, 1957). A most significant finding has been the depletion of CoA content (Severi and Fonnesu, 1956). The two most important compounds, adenosine triphosphate and CoA, are thus found to be reduced under conditions of accumulated pathological fat in the liver. It is significant that both are implicated in the fatty acid activating system. But whether the activating system affected is mitochondrial bound or extramitochondrial is not decided. In correlation with these observations it has been shown that the mitochondria from diabetic livers have significantly decreased oxygen uptake, phosphorylation, and P/O ratios during oxidation of pyruvate (Vester and Stadie, 1957). The livers from the animals were markedly fatty, whether as cause or effect of the oxidative distress is debatable. In view of recent findings that the essential fatty acids are required for structural stability and phosphorylative efficiency of mitochondria, it is necessary to keep in mind that drastic morphological and functional changes may be precipitated by rapid exhaustion of these sparse compounds (Levin, Johnson, and Albert, 1957).

Hormonal Control

The problem of abnormal control of tissue metabolism by hormones has been best exploited with excess thyroid activity. Total energy output is closely modified by graded stimulus of thyroid hormone and its derived active products. Only recently has an approach been achieved to the exact site of cellular activity under the influence of this hormone. From the early work of Maley and Lardy (1953) it was proposed that the active hormone and thyroxin might produce their effect by uncoupling oxidative phosphorylation, which in essence signified a purposeless oxidation of metabolites. DuToit (1952) had reached the conclusion that thyrotoxicosis is related not to control of individual enzymes but to an effect on the mitochondrion in the regions where respiration chains are coupled to the production of high-energy phosphate bonds. The subsequent investigations by Tapley and Cooper (1956) confirmed this view and indicated that the immediate action of the thyroxin and related active compounds was to increase mitochondrial volume and derange the mitochondrial processes concerned with control of fluid and electrolyte balance within the particle. These effects occur at thyroxin levels considerably below those required to uncouple oxidative phosphorylation. There is no question indeed but that mitochondrial morphology is considerably changed from normal in both hypo- and hyper-thyroid states (Abei and Abelin, 1953). It is suggested that the hormone performs its metabolic regulation by directing the attachment of critically placed magnesium ions which are known to stabilize mitochondrial configuration. One group has compared thyrotoxicosis with magnesium deficiency clinically and further demonstrated identical P/O ratios in heart mitochondria from both thyrotoxic and magnesium-deficient animals. It is known that magnesium has power to reverse the swelling potency of thyroxin in isolated mitochondria, and that hypothyroid patients excrete excessive amounts of magnesium after receiving thyroxin (Tapley, 1955). It may be suggested that the states of euthyroidism and departures from it into conditions of hypo- and hyper-thyroidism are mainly dependent on the balanced magnesium-thyroid control of mitochondrial structure and through this upon the rate of oxidative metabolism. Further support for this relationship is provided by the series of observations which establish a connexion between the stability of mitochondrial morphology and the binding of the magnesium and manganese ions by the mitochondria (Hunter and Ford, 1955; Bartley and Amoore, 1958), so that agents freeing them also loosen the mitochondrial hull and allow swelling and leakage of nucleotides to follow. The careful studies of Raaflaub (1953) and others on the stabilizing influence of adenosine triphosphate indicate that such ions are an essential co-constituent for the most satisfactory system. It is correct to presume that potassium binding is also a structural feature, which permits contractual relations between the mosaic of enzymes which operate in the oxidative phosphorylative mechanisms, whether the process is engaged at the total particle level or in subunits derived by special fractionation techniques (Gamble, 1957). The bound potassium therefore enters into the scheme for thyroxin activation. A preliminary report by Panagos, Beyer, and Masoro (1958) has shown that liver mitochondria are stress points in animals exposed to cold, and explain the resulting uncoupling of oxidative phosphorylation by intermediate action of increased thyroid secretion. The studies of cardiac mitochondria by Strawitz and Hift (1956) and by Hift and Strawitz (1958) with animals in irreversible haemorrhagic shock have demonstrated profound morphological, metabolic, and chemical alterations. Mediation by hormonal stress, including thyroid excess, is likely. As yet no examination of mitochondria under the influence of thyroid hormones has been made by

electron microscopy, though such a study is now an essential part of the survey at electron level.

Since the *modus operandi* of thyroid hormone and analogues has revolved around mitochondrial structure, it is pertinent at this juncture to explore a range of pharmacological and toxic compounds which affect metabolism at the same point of stress. A recent interpretation of the action of dinitrophenol, which stimulates oxidations and impairs phosphorylations, has attached considerable importance to the morphological deformities induced by dinitrophenol (Kaltenbach and Harman, 1955). Other investigations designed to clarify participation of a complex interrelation between *p*H, metal ion complexon formation, and thermal reactivity in adenosine triphosphatase activity have taken into consideration the importance of alteration in mitochondrial age (or structure) as a factor contributing to the shift from a mechanism which transfers energy through a high energy intermediate to adenosine diphosphate to one which allows the intermediate to dissipate before the transfer is effective (Kielley and Bronk, 1958). Damage is extended by the fall in ~P and the activation of adenosine triphosphatase which is normally cryptic, so that a rapid mitochondrial disintegration ensues and oxidative metabolism is unfettered. The attack by dinitrophenol on the mitochondrial phosphorylation sequence is thus a model of the basic pattern of injurious interference with this fundamental metabolic pathway. A wide variety of dyes and biological compounds have been so analysed (Harman and Feigelson, 1952).

Perhaps one of the most interesting studies has been on the behaviour of mitochondria and respiring tissue under the influence of excessive concentrations of several naturally occurring pigments. It was shown by Zetterström and Ernster (1956) that unconjugated bilirubin in quantities approximating the level seen in hyperbilirubinaemic syndromes associated with haemolytic disease of the newborn (Ernster, Herlin, and Zetterström, 1957) have a decisive uncoupling action on liver and brain mitochondria. The bilirubin causes not only a severe inhibition of the phosphorylation but also depresses oxidation, and manifests this in both liver and brain mitochondria. This property of bilirubin, in unconjugated form, has been linked by several groups with the occurrence of kernicterus, or, as it may be more accurately called, if the relationship holds fast, "bilirubin encephalopathy." Biliverdin and the conjugated bilirubin are incapable of uncoupling. These observations have been repeated with similar results and the range of substrates confirmed. It has been found, however, that inclusion of albumin with the mitochondria provides appreciable protection to the oxidative phosphorylation (Harman, 1958). Furthermore, bearing in mind that metabolic products or excessive accumulation of haemoglobin precursors may be injurious, the action of haematoporphyrin on liver mitochondria was determined. It provoked (Table I) morphological changes similar to cloudy swelling, uncoupled oxidative phosphorylation, and yet prevented the appearance of adenosine triphosphatase activity. The uncoupling activity was moderately reversed by albumin, as in the case of bilirubin excess. This

TABLE I
EFFECT OF HAEMATOPORPHYRIN ON LIVER MITOCHONDRIA

System	P:O	Cytology (Crescents %)
Glutamate	3·0	Normal
+ Albumin 3·5%	2·8	Normal
+ Haematoporphyrin	0	All degenerate
+ Both	0·9	50% degenerate
Malate	3·0	Normal
+ Albumin 3·5%	3·1	Normal
+ Haematoporphyrin	0	All degenerate
+ Both	2·43	21% degenerate

TABLE IIA
EFFECT OF DINITROPHENOL ON OXIDATIVE PHOSPHORYLATION IN ISOLATED MITOCHONDRIA WITH AND WITHOUT ALBUMIN
Substrate: succinate.

Additions	Without Albumin			With Albumin		
	ΔP μM	ΔO Micro-atoms	P : O	ΔP μM	ΔO Micro-atoms	P : O
None	19·4	11·3	1·7	25·6	13·0	1·9
DNP M/100	3·8	4·9	0·78	1·8	7·5	0·24
DNP M/1,000	1·2	9·5	0·12	29·8	14·2	2·09

TABLE IIB
EFFECT OF GRADED LEVELS OF DINITROPHENOL ON OXIDATIVE PHOSPHORYLATION IN ISOLATED MITOCHONDRIA WITH AND WITHOUT ALBUMIN
Substrate: oxyglutarate.

Additions	Without Albumin			With Albumn		
	ΔP μM	ΔO Micro-atoms	P : O	ΔP μM	ΔO Micro-atoms	P : O
None	20·4	9·0	2·2	22·8	12·3	1·9
DNP M/100	+1·8	3·5	—	+1·02	4·2	—
DNP 2×10^{-3}M	+3·7	4·2	—	20·4	8·8	2·3
DNP M/1,000 0·1 ml.	7·6	9·0	0·84	19·6	14·6	1·3
DNP M/1,000 0·05 ml.	10·4	9·4	1·1	21·2	14·0	1·5
DNP M/10,000	17·2	8·79	1·9	20·0	13·2	1·5

effect of albumin is at the moment under scrutiny (Table IIA, IIB), and it has been found capable also of neutralizing the uncoupling action of dinitrophosphatase on phosphorylation (Clarke, 1958). The neuronal damage in both these derangements of bile accumulations and abnormal porphyrin metabolism are explicable on the mitochondrial scale, but caution is advocated in view of the decidedly protective mediation of serum albumin at physiological levels.

Vitamin Control

For many years a relation between the tocopherols and muscle diseases, especially the dystrophies, has been favoured but fortified with only marginal evidence, which was often contradictory. The recent experiments by Nason, Donaldson, and Lehman (1957) have offered an escape from the dilemma of conflicting results and supplied a site for the intervention of the vitamin in the metabolism of muscle and other tissues. It is obvious from extraction studies with both homogenates and purified mitochondria suspension that the alphatocopherol is committed to participation in the respiratory chain. Removal of a lipid component from particulates of rat skeletal muscle, which corresponds approximately with a sarcosomal-mitochondrial admixture (Kitiyakara and Harman, 1953), interfered electively with electron transport at the site of disphosphopyridine-cytochrome c reductase activity. Although the enzymatic activity of the particulate DPNH-cytochrome c reductase is re-established by the addition of alphatocopherol, or the residue of the iso-octane extract, it can also be reconstituted by fortifying the extracted particles with 1% serum albumin. Since neither the extract nor the albumin contains alphatocopherol, the full composition of the co-factor is not defined and may be a complex containing alphatocopherol. Bouman and Slater (1957), however, established that an active Keilin–Hartree suspension of heart muscle, which is mainly composed of mitochondrial fragments, contains significant quantities of alphatocopherol, and reached the result that it is all mitochondrial bound. Slater (1958) suggested that it or a derivative quinone is an essential link between oxidation and phosphorylation. Further studies by the same group challenged the specificity of the tocopherol at enzyme level and postulated that the compound favourably reorientated physical properties (structure) dispersed by iso-octane elution. In accordance with the findings of Martius (1956), who suggested that the defect of vitamin E undermines oxidative phosphorylation, the above close analyses move the problem of muscular dystrophy closer to a solution.

The series of reactions as postulated from sundry studies depicts the hypothetical phosphorylation mechanism normally linked to the electron chain at several established sites (equations 1–4). It is

$$AH_2 + B + I \rightleftharpoons A\sim I + BH_2 \quad \ldots\ldots(1)$$

$$A\sim I + X \rightleftharpoons A + X\sim I \quad \ldots\ldots(2)$$

$$X\sim I + P_i \rightleftharpoons X\sim P + I \quad \ldots\ldots(3a)$$

$$X\sim P + ADP \rightleftharpoons X + ATP \quad \ldots\ldots(3b)$$

$$X\sim I + H_2O \xrightarrow{(DNP)} X + I \quad \ldots\ldots(4)$$

conceivable that the deficiency of tocopherol or a like quinone effects structural damage to the mitochondrion so as to promote hydrolysis of X~I A loss of respiratory control and lowering of P/O ratios would ensue, and the action in this instance would simulate that of dinitrophosphatase. It has been suggested alternatively that the quinone is actually X, although this is a less acceptable theory, and that its absence will allow accumulation of A~I which may suffer hydrolysis by an alternate mechanism, whereby oxidation of the substrate is unimpaired but the phosphorylation is restricted. Such a view would account suitably for rapid exchange of ^{18}O between water and PO_4, the inorganic phosphate of the system (Boyer). Recent analyses have indicated that the quinone functioning in the system may not be alphatocopherol but another compound designated as Q 275 (mitoquinone) by Crane, Hatefi, Lester, and Widmer (1957) and as ubiquinone by Festenstein, Heaton, Lowe, and Morton (1955). It is apparent from the studies by Slater (1958) that the bulk of the quinone in heart mitochondria occurs as ubiquinone or mitoquinone. Other workers (Hatefi, Lester, and Ramasarma, 1958) have reported in preliminary observations that the mitochondrial bound mitoquinone is rapidly reduced by succinate, DPNH, hydroxybutyrate, and diphosphopyridine nucleotide-dependent substrates of the Krebs's cycle, with specific inhibition by antimycin for succinate and amytal inhibition for the other substrates. Furthermore ubiquinone was able to restore activity to iso-octane-extracted particles. These findings favour the opinion that the quinone may be X in the phosphorylation scheme above. In either case the participation of the vitamin or its derivative in the metabolic pathway emphasizes its critical role in the maintenance of cell structure. Other workers concerned with the

disposition of energy generating and transport systems in the muscle cell had proposed that the critical lesion was to be sought primarily in the mitochondrial system of integrated enzymes (Harman, 1955). It is also worthy of mention at this juncture that several teams of investigators have reached agreement that vitamin K is also involved in the processes of oxidative phosphorylation in the mitochondria and that its focus of function is at approximately the same position as that of vitamin E.

Paramitochondrial Organelles

Both by electron microscopy and the separation techniques of differential centrifugation, families of particles within the volume and density range of the mitochondria have been categorized. The Belgian group under deDuve have pioneered this exploration and provided a nomenclature and characterization which in part is dependent on enzymatic criteria. The studies of Chantrenne (1950), of Laird (1954), and subsequently of Novikoff (1956a) have sharpened the delimitations between these units and the conventional mitochondria. In all instances the newly discovered particles have been distinguished by different ribonucleic acid content, an array of specific enzymes either absent from or of slight importance in the mitochondria, and finally by a lack of inner lamellar structure usual in the mitochondria (Novikoff, 1956b). The particles are especially rich in β-glucuronidase, cathepsin, acid phosphatase, ribonuclease, and desoxyribonuclease. It was considered, because of the hydrolytic nature of these enzymes, that they were contained in a distinct class of granule which has been provisionally named the "lysosome." It seems that just as the mitochondria occupy a pivotal position in the oxidative and phosphorylative metabolism of the cells, these lighter granules may similarly be storehouses of lytic enzymes. It is obvious at once that disturbance of such particles can unleash a most destructive series of enzymes and that the consequences are certainly pathological. Confirmation of this is afforded by the observations that acid phosphatase, β-glucuronidase, and cathepsin levels are considerably accentuated in fatty livers induced experimentally. Uricase concentration is shifted to finer particles by the administration of carbon tetrachloride. It may be inferred from such investigations that the damage incurred during the process of cloudy swelling and more advanced cytonecrotic lesions involves not only a direct interference with endergonic processes but activates mechanisms which directly destroy important cellular constituents.

Another interesting particle with special pathological interest has been identified in association with normal and deviated iron metabolism. Electron microscopy has revealed a characteristic structure for the ferritin molecule (Kuff and Dalton, 1957) and confirmed Farrant's (1954) demonstration of the micellar pattern of the molecule. Under normal conditions such particles are distributed uniformly throughout the cytoplasm, with a slight tendency to group more densely between adjacent mitochondria. With the infusion of large amounts of saccharated iron and haemoglobin the cytoplasmic concentrations were vastly augmented. Under such conditions the ferratin micelles appear in otherwise structureless particles of approximately mitochondrial dimension, at which time the reactions and chemical properties typical of haemosiderin may be elicited. Rupture of the bodies is believed to dump cohesive clumps of haemosiderin or catabolized ferratin into the hyaloplasm. The bodies which accumulate the ferratin (Fig. 13) and catalyse its

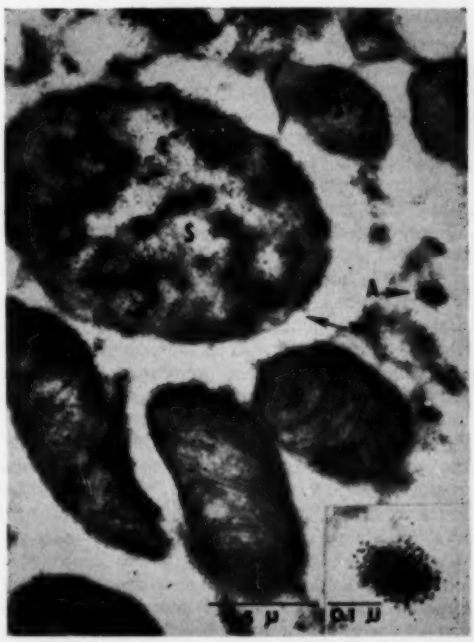

FIG. 13.—This is a photomicrograph of a siderosome as demonstrated by Richter (1957) from liver of an iron-overloaded rat. Some resemblance to the lysosome is apparent. His lower inset shows free ferratin granules indicated at A.

transformation into haemosiderin are termed "siderosomes" to distinguish them from both mitochondria and lysosomes. At a much earlier date others proposed that the haemosiderin particles were developed within mitochondria during a process of "cytosiderosis" which occurs either in a state of iron overloading or certain nutritional deficiencies. The introduction of electron microscopy, with its revaluation of the fine structure of organelles and of the iron particles, eliminates the mitochondria for the present and substitutes a special functional granule (Richter, 1957).

Numerous other cytoplasmic particles of a special nature have been known for many years, such as those in eosinophil and basophil cells, pituitary chromophil cells, and neutrophil leucocytes. Electron microscopy has revealed that some of these have a peculiar structure, but presents no advance in specifying function. The mast cell granules, however, have been correlated with the release from the cell under the impact of salmine and protamine, as well as of toluidine blue, of histamine together with the dissociation of the specific granules. Electron microscopy has satisfactorily defined this relationship which may be an important part of the inflammatory reaction.

Tissue Defence

It may be presumed from the fragility of the mitochondria and their vulnerability to noxious agents that they mainly represent disease targets. It is fascinating to discover in one type of cell, and perhaps in others if investigation were pressed home, that they can put the tissue into the attack. Fishman and Silverman (1957) analysed the digestive phase of phagocytosis. Suspensions of polymorphonuclear leucocytes were disintegrated by conventional Potter-Elvehjem homogenization and the homogenate partitioned into fractions, including the mitochondria, although no mention is made of chromophilic granules. The various particles are then dispersed by sonic vibrations and the extracts tested for bactericidal activity. The mitochondrial fraction contained the bulk of the activity, with none evident in the soluble hyaloplasm. The mitochondrial extract was five times more concentrated than the extract from the total cell mass. Characterization demonstrated the lipoprotein nature of the active component, which may be derived from the inner lipoprotein lamellae of the particles. A correlation was indicated between the effectiveness of the bactericidal agent against various microbial strains and the natural invasiveness in the species studied (Fishman, Cole, and Silverman, 1957).

Cytochondria and Carcinogenesis

The subcellular metabolic and structural patterns of neoplastic cell populations have been defined by several schools concerned especially with the cytoplasmic particulates. It is the pattern of cellular organization rather than absolute quantitation which has received focal interest. Laird (1954) has determined the protein and ribonucleic acid distribution in the particulates and soluble fractions of numerous tumours and normal cells and describes distinctive patterns for the neoplasms. It was also well established that the mitochondrial population of preneoplastic and neoplastic tissues is decreased, due to diminution in number, occasionally in size, and often in structural density of the units (Allard, de Lamirande, and Cantero, 1953). In chemically induced hepatoma the count per cell may be halved, and there is a greater decrease in many individual oxidative enzymes per gram of mitochondrial protein than might be expected from the simple population change. This is compatible with the observation that the oxidative phosphorylation of hepatoma is marginal and also excessively fragile under stress (Emmelot and Bos, 1955). Mitochondria isolated from tumours lose their phosphorylating capacity very rapidly compared with control tissues, although supplementing with diphosphopyridine nucleotide provides almost complete restitution. Wenner and Weinhouse (1953) had previously estimated the diphosphopyridine nucleotide content of hepatoma mitochondria and emphasized the paucity and loose binding of the co-enzyme in the neoplastic particles. Carruthers and Suntzeff (1954) attributed this scanty co-enzyme reserve to an excessively active nucleotidase in the tumour mitochondria. In either instance it is also likely from our understanding of normal mitochondrial stability that the ATP-ADP pool and its maintenance participate in the conservation of co-enzyme and that this phosphorylating system is primarily defective.

It is unlikely that the mitochondrial behaviour as such is involved directly in the causation of cancer. Recent information concerning mitochondrial formation *de novo* in actively dividing cells may afford insight into the deviations of mitochondrial pattern in tumour cells, which are also characterized by nuclear abnormalities. Hoffman and Grigg (1958) have advanced compelling evidence that the mitochondria are formed from the nuclear membranes during the mitotic cycles in several normal cell types. Earlier work by Hartmann (1948) illustrated the occur-

rence during chromatolysis of an increase in the mitochondrial population of nerve cells, which Causey and Hoffman (1955) subsequently associated with the specific nuclear alteration and mitochondrial attachments, by electron microscopy. Although this hypothesis is not exclusive and permits alternative variants of mitochondriogenesis, such as fission and derivation from regeneration microbodies after initial fragmentation according to Gansler and Rouiller (1956), they propose it as the chief mode of mitochondriogenesis. Rouiller and Bernhard (1956) observed a great increase of dense microbodies in hepatoma cells, in which they are concentrated adjacent to nuclear and cell membranes.

The defect in mitochondrial reproduction and structure may sequentially influence the ergastoplasm with its gamut of chromidial points and ribonucleic-acid-rich particles. Arcos and Arcos (1958) have advanced information concerning the modification of the macromolecular organization of the microsomes when inducing chemical carcinogenesis. Although they prefer to regard the alterations in the ergastoplasm (microsomal pellets) as the major participant in carcinogenesis, the observations by Bernhard and Rouiller (1956) that mitochondria govern ergastoplasmic formation, and the finding of Clerici and Cudkowicz (1956) and Emmelot and Bos (1955) that the mitochondria are specifically altered during carcinogenesis, prevent full acceptance of their thesis that the microsomes are more directly involved in carcinogenesis than other cell particles. Observations by Waravdekar, Powers, and Leiter (1956) on sarcoma 37 illustrated a gross increase in drug sensitivity of oxidases contained in the mitochondria. From the various studies of particulates from neoplastic cells it is clear, however, that they all share the disturbed functional and structural abnormality of the cell, especially at the level of intermediate metabolism.

Microsomes

The microsomes are situated in the endoplasmic reticulum as the units of an intertwining cytoplasmic network. Although a tendency to ramification of the strands is apparent in some cells the extension through the cytoplasm is random. Functionally, the constituents of this chain-like system of vesicles are concerned with many of the cell's synthetic mechanisms, deriving energy from the mitochondria and the substrate from compounds activated by enzyme systems in the hyaloplasm. They are especially rich in ribonucleic acid, which is a recognizable feature in their identification. Thus far the few diseases which are specific to them arise from deficiencies in their enzyme systems.

The microsomes are implicated in two major parameters of cytoplasmic activity. They participate in pathways of protein and steroid synthesis and secondly are responsible for a broad spectrum of reactions concerning detoxication and with conjugation mechanisms. In the first category of synthesis the particles, or the ramifying vesicular system of the endoplasmic reticulum, have a direct controlling influence on protein synthesis, independent of the remote, genetic regulation by the nucleus. Numerous experiments with enucleated cells have verified this. It is the ribonucleic acid moiety of the microsomes which influences protein synthesis in a specific manner so that specific ribonucleic acids induce elaboration of particular proteins and enzymes (Minagawa, 1955). After the amino-acid residues are activated in the presence of soluble ribonucleic acid (Hoagland, 1955) with formation of aminoacyl adenylates they remain firmly attached to the activating enzyme until the residue is incorporated rapidly into the ribonucleic acid of the microsome, which is known to incorporate the amino-residues more vigorously than all other cell fractions as discerned in Graph No. 2 of Siekevitz's (1952) analysis. This step is a most critically orientating movement which precluded random reaction between the highly reactive anhydrous aminoacyl adenylates with formation of well-defined but heterogeneous proteins (Chantrenne, 1950). It is believed that guanosine phosphate mediates the critical step of incorporation into the microsomal ribonucleoprotein (Hoagland, Zamecnik, and Stephenson, 1957). Thereafter the attached amino-acids are folded into the correct configuration according to one of several possible template mechanisms with the formation of a genetically pre-established configuration (Da'gliesh, 1957). It is most obvious that this is a fertile area for pathological deviation.

The acute interference with the synthetic mechanism, whether by deprivation of an essential amino residue or intervention with an analogue during activation, allows formation of a defective protein, or precludes elaboration of normal protein. On the other hand, under the impetus of more general stress the ribonuclease may be liberated from the nucleus and by participating in the depolymerization of ribonucleic acid impede the incorporation of activated moieties by the particles and cause a general impairment of protein synthesis. This is manifest after toxic

ionic irradiation when both nuclear ribonuclease is released and mitochondrial structure damaged (Okada and Peachey, 1957). It might be recalled that the phenomenon of cloudy swelling is accompanied by a depletion of the basophilic (ribonucleic acid) constituents of the cell (Drochmans, 1947). In a more chronic form the microsomal synthesis may be impressed into abnormal patterns either by genetic abnormalities in the template construction or the inclusion of metabolic analogues to supplant the normal residues. Some attempt has been made to exploit this hypothesis in tumour chemotherapy. The analogue 3-thiophenealanine causes regression of rat sarcoma transplants and inhibition of antibody formation, with reversal of the effects by phenylalanine (Wissler, Frazier, Soules, Barker, and Bristow, 1956). Similar antimetabolites, including beta-2-thienylalanine, interfere with protein and nucleic acid metabolism and furnish evidence for the likelihood of this occurring in teratogenesis and congenital abnormality (Ham and Eakin, 1957). Studies on urchin eggs' development have been most rewarding with their revelation of intervention of amino-acid antimetabolites in radialization of the organism (Gustafson and Hörstadius, 1955). In the same sphere it has been suggested that differential supplies of simple metabolites may profoundly affect the protein synthesis, as each phase of development has specific quantitative requirements. It is salient that not only protein synthesis is subject to derangement, but that the functioning enzymes which they comprise in most instances are also disorientated, so that the pathological effects are accumulative. Holtfreter (1955) has presented provocative observations on the pathogenic properties of "inductive" agents derived from dead tissue, with exhibition of micro- to acephalic hypomorphoses. Random cell death during morphogenesis may be, at critical stages, as injurious as destruction of select cell groups.

The participation of the microsomes in protein synthesis is a general phenomenon in all cells. Its importance in pathogenesis is well illustrated in two special instances. The first concerns formation of haemoproteins in erythrocyte precursors on the one hand and in parenchymal cells of several tissues on the other. It was demonstrated that particulated fractions from reticulocytes and other earlier red forms, with a high ribonucleic acid content, rapidly incorporate activated amino residues under added stimulus with folic acid and vitamin B_{12}, first into the microsomes and subsequently into the globin (Rabinovitz and Olson, 1956). This is illustrated in Fig. 14 which corresponds with observations now conventional for liver muscle and red cell microsome activity. Arnstein has directed attention to the effectiveness of vitamin B_{12} in abetting the protein synthesis of many cell types as well as the erythropoietic series, although the deficiency of this factor is especially salient in erythropoiesis because it simultaneously affects metabolic reactions involved in haem and porphyrin synthesis. Rabinovitz and Olson (1958) also establish the intervention of microsomes in the incorporation of iron into haemoglobin. The synthesis of haem compounds also requires the active intervention of mitochondria in parenchymal cells as manifested in studies of the introduction of iron into the haem by rat liver particulates. It is inferred from this that the timing of haem and protein synthesis in cellular maturation may be sequential and under the influence of cell particulates. In support of this it is known that in erythropoiesis the mitochondria concerned in synthesizing the cell mass and haem disappear somewhat early, whereas the ribonucleic-acid-rich microsomes which direct globin formation and iron inclusion are seen as late as the reticulocyte stage. It is apparent that closer analysis of pathogenesis in deficiency and degenerative anaemias now requires study of the particulates in red cell precursors.

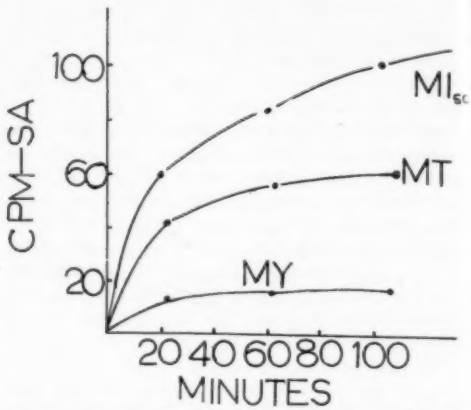

FIG. 14.—This exemplifies the type of activity observed with microsomes from liver, reticulocytes, and parenchymal cells of all types. The preparation utilized consists of particles from skeletal muscle representing micro-sarcosomes (Mi_{sc}), mitochondria (MT) and myofibrils (MY). In this tissue mitochondria manifest more than usual activity. Uptake of labelled leucine was measured. Mitochondrial uptake is considerable.

The second special instance in which the microsomes are associated with deviated protein synthesis occurs in the binding of carcinogenic azo

dyes by the liver cell proteins. Miller and Miller (1947) have concluded on the basis of important considerations that this binding process may be engaged in tumour induction. The preneoplastic maxima of dye binding were closely correlated with specific cytoplasmic hyaline inclusions characteristic of the azo dye intoxication (Price, Harman, Miller, and Miller, 1952). It has subsequently been shown by Hultin (1956) that in early stages of azo dye fixation during maximal hyaline plaque formation the dye is predominantly part of the microsomal protein complex. It is subsequently transferred to soluble or extramicrosomal pools which precipitate in the cytoplasm to form the characteristic plaques. Such protein development may constitute an important aberration in microsomal metabolism and structure as indicated by Arcos and Arcos (1958), who followed the levels of bound dyes in the microsomes and correlated them with altered microsomal structures. In later work Hultin (1957) also detects two species of microsomes manifesting differential binding, and incorporation capacities may in part explain some of the results obtained by these workers. But it is apparent that the carcinogenic activity of the dyes is mediated in some manner through the metabolic processes of the microsomes concerned in protein synthesis.

It so happens also that the microsomes are the site of cholesterol and general sterol synthesis. The mitochondria may contribute high energy for activation, but definitive enzymes are situated in the microsomes. After activation with co-enzyme A and ATP the moiety is incorporated into the microsomes. The initial component may be lanosterol, with further conversion to cholesterol, which requires a TPNH reaction dependent on supernatant enzymes. Perhaps one of the most significant observations in this field is that the application of ribonuclease blocks the utilization of mevalonic acid in the synthesis of cholesterol (Wright, Cleland, Dutta, and Norton, 1957). This brings the synthetic pathways of proteins and the sterols into close conjunction, and may well be scrutinized for aspects of lipoprotein aberration, since this is the proper site for the appraisal of the basic defect in lipid storage diseases.

In its second parameter of activity the microsomal system has the capacity to oxidize and conjugate a wide variety of drugs and extraneous compounds. Both mechanisms have been classified as detoxicating processes, and a list of the types of reactions involved in oxidative detoxications is presented in Table III. Non-specificity is a characteristic of these enzyme mechanisms, which

TABLE III

Hydroxylation of aromatic rings

$$CH_3\text{–}CO\text{–}NH\text{–}C_6H_5 \xrightarrow{(OH)} CH_3\text{–}CO\text{–}N\text{–}C_6H_4\text{–}OH$$

Oxidation of side chains

$$R\text{–}CH_2CH_2CH_2\text{–}CH_3 \xrightarrow{(OH)} R\text{–}CH_2CH_2\text{–}CH_2OH\text{–}CH_3$$

N-Dealkylation

$$R\text{–}NH\text{–}CH_3 \xrightarrow{(OH)} (R\text{–}NH\text{–}CH_3OH) \xrightarrow[\text{Unstable}]{} R\text{–}NH_2 + HCHO$$

O-Dealkylation

$$R\text{–}O\text{–}CH_3 \xrightarrow{(OH)} (R\text{–}O\text{–}CH_2\text{–}OH) \xrightarrow[\text{Unstable}]{} R\text{–}OH + HCHO$$

Deamination

$$R\text{–}CH_2\text{–}CH((NH_2))\text{–}CH_3 \xrightarrow{(OH)} (R\text{–}CH_2\text{–}COH((\text{–}NH_2))\text{–}CH)_3) \xrightarrow[\text{Unstable}]{} R\text{–}CH_2\text{–}CO\text{–}CH_3 + NH_3$$

Sulphoxide formation

$$R\text{–}S\text{–}CH_3 \xrightarrow{(OH)} (R\text{–}S\text{–}CH_2\text{–}OH) \xrightarrow[\text{Unstable}]{} R\text{–}SO\text{–}CH_3$$

are not critically engaged in normal sequential metabolism, so that deviation to handling foreign compounds does not deplete the organism of essential enzymes. They have a further special characteristic feature of acting mainly on lipid-soluble non-polar compounds. In mammals this is of considerable importance in order to prevent accumulation of toxic lipid-soluble compounds which might tend to concentrate in dangerous quantity. Metabolism of compounds with high oil-to-water partition is probably associated with the lipid cortex of the microsome which can be penetrated only by suitable non-polar molecules. This is an important arena for the experimental pathologist in so far as the competence of these systems is dependent on an adequate range of enzymes. Sex and species differences have been discerned and microsomal variation may be the explanations for such metabolic difference in handling compounds. It is conjectural that certain instances of drug hypersensitivity pertain to the efficiency of this system.

One of the most fascinating recently observed diseases is due to a defect in this system which is responsible for constitutional non-haemolytic hyperbilirubinaemia (Gilbert's disease). It is ascribed to a failure by the liver microsomes to conjugate bilirubin with glucuronide, which is the soluble direct form and susceptible to excretion. Failure in conjugation prevents adequate excretion and establishes a hyperbilirubinaemic state. With the use of a special mutant strain of Wistar rat, afflicted with this syndrome, it was found that the microsomal fraction lacks a glucuronyl transferase, which shifts the glucuronide from uridine-diphosphate-glucuronic acid to the bilirubin

molecule (Schmid, Axelrod, Hammaker, and Swarm, 1958). It is a general defect in glucuronyl transfer as shown with the use of o-aminophenol as acceptor. Later work has extended the existence of such a deficiency to foetal and newborn livers, where it assumes considerable significance in relation to the mechanism of icterus which is so frequent at that age. Any abnormal tendency to haemolysis will be an overload for foetal and newborn livers, and remarkably dangerous in view of the suspectibility of the foetal neurons to bilirubin encephalopathy (Zetterström and Ernster, 1956). It is likely that variable degrees of this and kindred so-called "detoxicating" mechanisms may account for types of cytoplasmic sensitivities to extraneous compounds at subliminal concentrations.

Summary

In the hundred years since the publication of Virchow's classic synthesis on cell pathology the orientation provided by that definitive work has been sufficient. It has also indicated the logical further step into the field of cytoplasmic nutrition and function which govern the basic cellular reactivity in health and disease. The concept of the cell as a nutritional unit suggested the manner in which the cell suffered damage while reacting to diverse forms of injury or malnutrition, but realization of the mechanisms engineering this damage within the cell was denied owing to the inadequacy of extant knowledge concerning cytoplasmic structure and function then current. Within the last decade the panorama of subcellular structure and function has opened up before us, thrusting upon the pathologist the novel task of pressing beyond the cellular into the subcellular range of disease. In this advance of cellular pathology to a new level the acceptance of the particulate structure of cytoplasm has allowed recognition of subcellular differentiations which serves to localize the finer sites of disease processes.

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TWO BIOCHEMICAL ASPECTS OF CELL NECROSIS

BY

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In 1866, Hoppé Seyler was appointed to the Chair of Physiological Chemistry at the University of Tübingen, eight years after Virchow had published his lectures on cellular pathology. Buchner was no more than 6 years old at the time. Biochemistry, as we understand it, did not exist. To-day the dynamic approach which is the essential feature of modern biochemical teaching has produced advances in every biological field into which it has permeated.

The technical advances of the last three decades have given a much clearer picture of the anatomy of the giant molecules of which the living cell is built and through which it carries on its normal functions. Moreover, it is now possible to study to what extent and how fast the smaller units of which these giant molecules consist are built into the larger structure. This, if one likes to think of it, is anatomy and physiology at a molecular level. The natural extension of such studies is the examination of disease processes at a molecular level. By that one does not mean the movements of the electrolytes or the accumulations of such small units as urea or glucose, important as these are, but the changes in the character and concentration of the molecular units which comprise the cell itself. This is the logical extension of Virchow's cellular pathology.

In the general study of the cell's economy at molecular level one of the most active sections and one that is yielding the most rapid returns is the field of enzymology. Because some of the advances in this field have had direct clinical implications and because they are linked through histochemical studies to formal morbid anatomy it seemed apposite on this occasion to touch on this subsection of biochemistry. Two groups of enzymes, which will be familiar in name at least to all pathologists, have been picked at random for discussion. It is assumed that enzymes circulating in the serum have been elaborated in the cell and that with certain reservations fluctuations in serum levels mirror cellular changes. Indeed, Waterlow (1952) has produced some direct evidence to show that fluctuations in concentrations of circulating pseudocholinesterase parallel their concentrations in liver tissue.

However, two points must be borne in mind. It is obvious that one cannot assume that the changes in concentration in the circulation and in the tissue coincide. Moreover, if the apparent fall in circulating enzyme activity is due to an inhibitor incapable of penetrating the "cell membrane" there will be a discrepancy between cell and circulation so long as that inhibitor is effective.

The Transaminases

The transaminases are a large group of enzymes occurring throughout animal and plant tissues. They have interested biochemists for the last 20 years. Some 18 of these transaminases have been identified, of which two, the glutamate aspartate transaminase, more commonly called "glutamate oxalacetic transaminases" (G.O.T.), and the glutamate alarine transaminases, more commonly called "glutamate pyruvate transaminases" (G.P.T.), are of particular interest to the chemical pathologist. The chemical reactions involved are shown in Fig. 1. Both enzymes required phosphopyridoxal as a co-factor. Cohen and Hekhuis noted the high concentration of G.O.T. in cardiac muscle as long ago as 1941. But it was not until 1955 that LaDue and Wróblewski noted that when the heart muscle was injured there was a release of sufficient glutamate oxalacetic transaminase to produce a rise in blood levels.

Table I shows the relative concentrations of glutamate oxalacetic transaminase in tissue from data of Dubach (1957). It will be seen that myocardium is twice as rich in the enzyme as the liver or skeletal muscle and 50 times as rich as lung tissue. However, since skeletal muscle represents

TABLE I
CONCENTRATIONS OF GLUTAMIC OXALACETIC ENZYME IN TISSUES

	Units per g. of Homogenized Tissue
Myocardium	300,000
Liver	159,000
Skeletal muscle	135,000
Kidney	83,000
Testicles	78,000
Brain	38,000
Lungs	6,500
Bone	None found

From data of Dubach (1957).

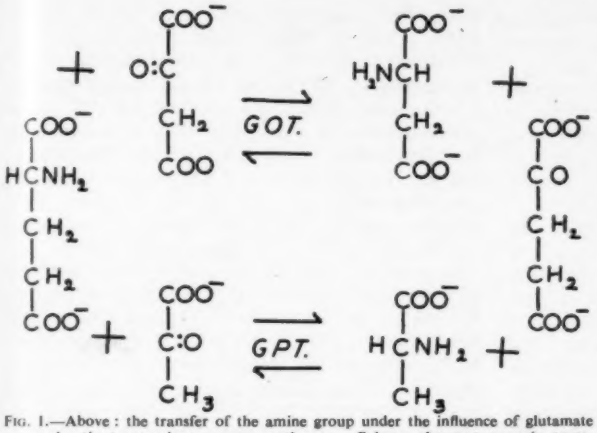

FIG. 1.—Above: the transfer of the amine group under the influence of glutamate oxalacetic transaminase, upper pathway. Below: glutamate-pyruvic transaminase—lower pathway.

about 40% of the total body weight it is, in practice, the richest reservoir of the transaminase enzyme. Indeed, in the "crush syndrome" seen so commonly in the war of 1939 to 1945, which resulted from extensive necrosis of voluntary muscle, it is very probable that levels of circulating transaminase were very high in the 48 hours following injury. Shabetai, Iglauer, and Anderson (1957) record one patient with acute pancreatitis who had a serum amylase level of 1,600 units and a serum transaminase level of 238 units.

Fig. 2 shows a plot of the level of the glutamate oxalacetic transaminase following acute myocardial necrosis of varying degrees of severity from our own material and from the data of Shabetai *et al.* and of Dubach. It will be seen that within 24 hours of myocardial damage there is a significant rise in the level of the circulating enzyme which is usually maximal by the second day, falling away rapidly again on the third or fourth day. Usually the circulating level has dropped to normal by the sixth day if there is no extension of the necrotic process.

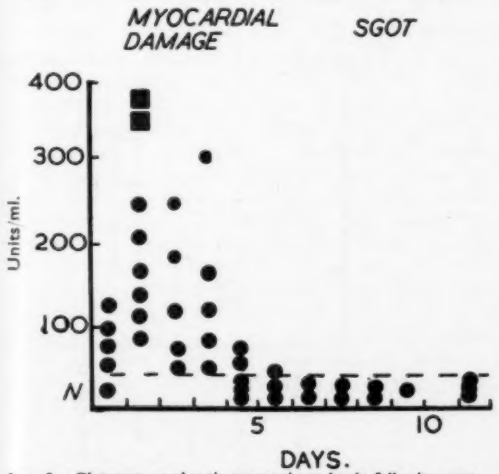

FIG. 2.—Glutamate oxalacetic transaminase levels following acute myocardial infarction. The black squares record two deaths.

If one assumes that the normal level of circulating G.O.T. is 40 units per minute, then the destruction of 1 g. of myocardium should raise the level of circulating transaminase to about 80 to 100 units per minute. Moreover, the destruction of a fifth of a gram should produce a detectable rise in the level of the circulating enzyme. That is roughly equivalent to 0.1% of the total mass of the heart. Naive calculations of this type are fraught with error, but they are useful if they stimulate thought.

Where it has been possible to make observations it appears that significant rises in circulating transaminase level do not occur much within 10 hours of the primary myocardial insult. Chinsky, Shmagranoff, and Sherry (1956) claim that 50% of patients with levels about 200 units die in the attack. The experimental work of Nydick, Wróblewski, and LaDue (1955) suggests that unless the myocardial ischaemia is sufficient to produce necrosis no rise in circulating transaminase can be demonstrated.

Where values have returned to normal, fresh infarction may be associated with a second rise in the level of the circulating enzyme, and it is this as well as the help the estimation gives in assessing various forms of retrosternal pain that gives this estimation its special value.

Here then is an example of an enzyme study which may from time to time be of very real use in the practice of medicine.

In the table of tissue concentrations it was noted that the liver was another organ rich in the glutamate oxalacetic transaminase. The liver is also rich in the glutamate pyruvic transaminase, another member of the same enzyme group. It is not surprising that recent studies of serum transaminase levels in various types of acute liver damage suggest a close relationship between the two. Fig. 3 shows serial studies of the serum levels of both enzymes during the course of attacks

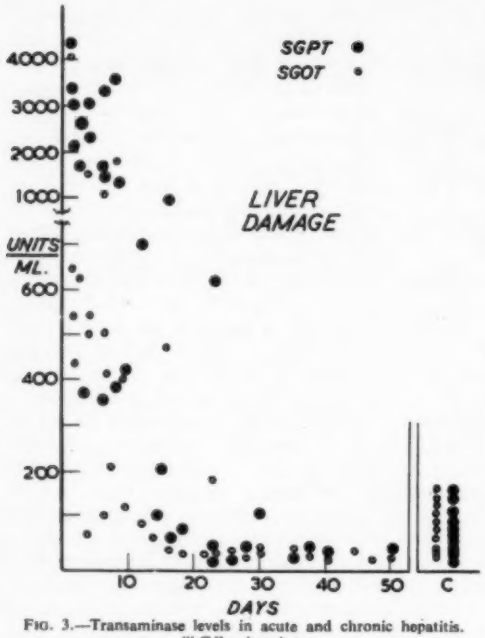

FIG. 3.—Transaminase levels in acute and chronic hepatitis. ("C" = chronic.)

of infective hepatitis from data by Henry in the St. George's Hospital laboratory. It is evident that the levels reached of the glutamate pyruvic acid enzyme are much higher on average than of the glutamate oxalacetic enzyme. Moreover, the levels recorded are already very high in the earliest stages at which clinical diagnosis is normally feasible and fall only gradually over the next 10 to 30 days.

Chinsky, Wolff, and Sherry (1957) demonstrated that the serum glutamate pyruvic transaminase levels were not elevated in myocardial damage in contrast with various forms of acute liver damage. Pryse-Davies and Wilkinson (1958), in a careful study of the diagnostic value of transaminase values in liver damage, found that they were raised in the early stages of hepato-cellular damage regardless of aetiology. They quote a patient who underwent a partial hepatectomy whose transaminase level was 800 units immediately following section, falling almost to normal limits by the sixth post-operative day.

Henry tested the sera from a number of patients with acute hepatitis and some who were known to have chronic liver damage (Fig. 3). The estimates were made when the patients were not in acute clinical relapse. It will be seen that the values were often raised, but never to the levels seen in acute hepatitis. Henry, like Pryse-Davies and Wilkinson (1958), found occasional normal values in cirrhotics. We would like to suggest that in chronic liver damage the elevated S.G.P.T. levels are indicative of a recrudescence of active liver cell destruction, whereas the normal values indicate a "resting" phase. If this view is correct, in following through a patient with relapsing progressive hepatitis we should observe a coincident waxing and waning of the transaminase level.

It is worth stressing that some of the methods of estimation are not highly specific and tend to fall off in their quantitative accuracy at ranges much above 1,000 units/ml. They seem perfectly adequate for routine chemical pathological practice. Pryse-Davies and Wilkinson (1958) have pointed out the effect of temperature on quantitative values obtained.

These then are two enzymes of a large and widespread group of intracellular enzymes which are sufficiently robust to survive the death and disintegration of the cells in which they normally function, enzymes whose release and time of survival in the blood stream give useful clinical information.

The Serum Cholinesterases

The detailed comparison of the red cell cholinesterases and the serum cholinesterases was made by Alles and Hawes (1940) 18 years ago. Adams and Whittaker (1949) and Whittaker (1951) compared the substrate specificity of the two groups of enzymes and on the basis of their findings proposed the name "butyro choline

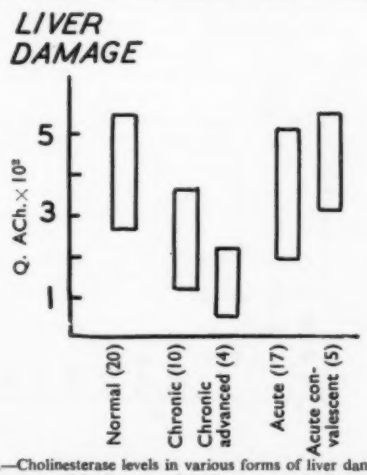

FIG. 4.—Cholinesterase levels in various forms of liver damage. (Q = enzyme activity in terms of total serum protein nitrogen.)

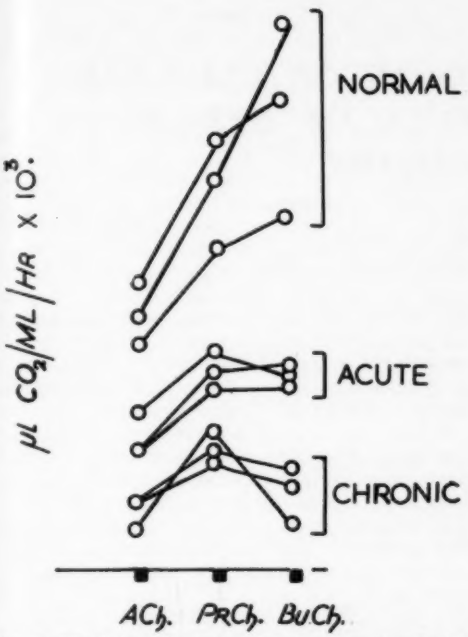

FIG. 5.—The differential esterase substrate pattern in acute and chronic liver disease after Kekwick (1955).

esterase" for the serum esterase commonly referred to as pseudocholinesterase. McArdle in 1940 had shown that the levels of serum cholinesterase decreased in patients suffering from liver damage, and many workers have confirmed his observations. Fig. 4 tabulates some results obtained by Kekwick (1955) when he was working on the esterases and their purification four years ago at St. George's Hospital. It will be seen that, unlike the transaminases, liver damage is associated with a drop in the levels of circulating "butyro esterase" activity and that this drop is most striking in patients with advanced chronic liver damage. It occurred to Kekwick to examine whether the fall in activity was associated with a change in the substrate pattern which Whittaker and his co-workers had described. Fig. 5 shows some of the results. There is an alteration in specific substrate pattern. This alteration could be the result of selective failure of the liver to produce a specific enzyme, the original pattern in fact representing a composite of enzyme activities, or that, as a result of continuous insults, the liver cell is failing to produce the "butyro esterase" in its final normal configuration, and releasing instead a "broken-backed" enzyme, the product of an incompetent cell. We know that in liver damage there is a quantitative failure: does this work of Kekwick's suggest a qualitative failure?

These are only two examples of the practical and potential value of enzyme studies in disease processes. There are many more—the phosphatases, the lipases, the dehydrogenases, to mention only a few. As our detailed knowledge of protein structure develops we shall undoubtedly be able to develop more exact knowledge of the mechanics of enzyme behaviour, for it is important to remember that to a large extent the enzymologist is not studying structure directly so much as behaviour patterns. He is the molecular psychologist. The specific capacity to hydrolyse an ester or group of esters is a function of an area and not of the whole molecule, though the integrity of the whole may be essential if the individual activity is to be maintained at a rate that enables it to be correctly integrated in the total activity of the cell.

Failure of integration is one aspect of the disease process as surely as any morphological change observed by histological techniques. But there is a long road ahead. As Kossel has said, the amino-acids that compose the protein molecule are as numerous as the letters of the alphabet and these are capable of expressing an infinite number of thoughts.

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EXTENSION OF THE LIMITS OF CELLULAR PATHOLOGY: THE ROLE OF ENZYME HISTOCHEMISTRY

BY

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"If we would serve Science we must extend her limits, not only as far as our own knowledge is concerned, but in the estimation of others."—R. Virchow (1858).

"Cytology cannot be content to go on indefinitely finding new visible parts of the cell . . . what it needs is to discover methods that will describe the organization of cellular events. . . ."—J. Z. Young (1956).

"New methods must be invented that will be sensitive enough to detect early functional changes."—G. R. Cameron and S. Muzaffar Hasan (1958).

My thesis is a simple one, and the three quotations which I have used, a selection from many which express the same opinions, illuminate what I have to say with a commendable economy of words.

The limits of cellular pathology, drawn up by Virchow 100 years ago, have been pushed forward in precisely the way that he suggested, by the application of histological techniques. At that time cellular pathology was not truly cellular but histological rather than cytological. Few pathologists are sanguine enough to foresee further extension of pathology by these same histological methods, and it is clear that if advances are to be made in our knowledge of the mechanisms of pathological processes then new methods must be invented which will "describe the organization of cellular events" and which "will be sensitive enough to detect early functional changes."

Although Virchow was content to state that permanent advances in medicine were made solely by "discoveries concerning the structure of the body," the rise of biochemistry, in particular, has made it clear that analysis of structure without function is relatively barren. Histochemistry is concerned particularly in the correlation of structure with function.

We cannot be satisfied, says J. Z. Young, with identifying the new parts of the cell even by histochemical methods, and it must be admitted that information derived from the application of methods of analytical histochemistry is in most cases of limited value. It is to the newer techniques of functional histochemistry that we must look for help, and it is these alone which can transform descriptive analytical histopathology into a new science of functional cytopathology. This thesis I shall try to develop briefly, and I shall illustrate it, for the sake of simplicity, solely with examples from the field of enzyme histochemistry. Before doing this, however, it may be expedient to give details of the present status of that youngest division of the relatively old science of histochemistry.

If we except the few historical methods for indophenol (cytochrome) oxidase, peroxidase, and the still current method for DOPA-oxidase, which were in existence in the early years of the century, enzyme histochemistry can be considered to date from 1939 when George Gomori first described his method for alkaline phosphatase. By 1953 histochemical methods had been evolved for the demonstration of 18 different enzymes. Most of these belonged to the hydrolytic group and the majority were either carboxylic acid esterases or phosphatases. To-day the figure stands at 45.

This is admittedly not very imposing when considered against the grand total of some 700 recorded enzymes (Dixon and Webb, 1958), but the distribution of these 45 methods among the main enzyme divisions is now much more even than it was only a few years ago. This is chiefly due to the development of methods for hydrogen transferring enzymes, particularly the dehydrogenases and diaphorases.

Applied Enzyme Histochemistry

A considerable amount of work has been carried out by the application of histochemical enzyme techniques to problems in the field of cellular pathology. Often the techniques them-

selves have been criticized for lack of specificity and sensitivity and for inaccuracy in localization. Many of them clearly need improvement on this account, but until such time as better methods are developed there is much to be said for the employment of existing methods if these can be shown to provide useful information. Thus, although the degree of localization provided by the standard techniques for alkaline phosphatase is less than one would desire, Novikoff (1955) remarks that it is difficult to see how the distribution of this enzyme within organs could have been studied without a staining method. Precisely the same may be said for other enzyme systems, but the mapping of these, however attractive and important it may be to the pure histochemist, has little appeal to the pathologist who wants to know how and why different processes occur as well as where and when.

There are, nevertheless, a number of indications for the use of histochemical enzyme techniques in pathology, and a few examples may be cited to illustrate some of the facets of their use.

General Enzyme Histochemistry

In spite of the fact that a reasonable explanation for the mode of action of the enzyme is seldom forthcoming, the alkaline phosphatase method continues to be one of the most popular methods for application to pathology and to the other basic sciences. A histochemical enzyme method may be useful, even if the mechanism of action of the enzyme is not understood, since it can act as a sensitive indicator of functional changes. Histologists have long sought some indication of the activity of the adrenal cortex other than simple fat staining. Recently, Allen and Slater (1956) have shown that the level of alkaline phosphatase in the connective tissue and blood vessels of the mouse adrenal cortex rises after stimulation of the gland with A.C.T.H. Similarly, Clayton and Hammant (1957) have observed an overall diminution of alkaline phosphatase in the cells of the guinea-pig adrenal cortex after A.C.T.H. In human adrenal cortical tumours those regarded as clinically active are found to consist of cells having little or no alkaline phosphatase, with an intensely active stroma and blood vessel walls. Thus the results of a simple enzyme technique may be used to evaluate activity in respect of corticosteroid production.

While a raised level of alkaline phosphatase in a barrier tissue such as a blood vessel wall usually signifies that the transmitting function of the barrier is increased, no such interpretation can be put upon changes in acid phosphatase levels. Several other types of activity, however, have been shown to be accompanied by a rise in the level of this enzyme. It has been shown to occur, for instance, in active phagocytosis (Grogg and Pearse, 1952) and in cytolysis (Green and Verney, 1956). A fall in the level has been found in most types of prostatic tumour (Woodard, 1952), and this finding is common enough in respect of many enzyme systems in many different kinds of tumour for it to have almost the force of a general law. That is to say, an enzyme found in large amounts in the normal parent cell of a tumour is likely to be present in reduced amounts in its malignant offspring. The main exceptions to this rule concern the respiratory enzymes, but apparent exceptions may occur where an enzyme absent from the presumed parent cell is present in the cells of the tumour. The probable peptidase found in carcinoid tumours by Pearse and Pepler (1957) falls into this category.

On the whole, acid phosphatase is best used histochemically as an indicator either of increased mitochondrial activity or of actual mitochondrial damage, since a diffuse reaction in properly fixed tissues means that the enzyme has escaped from its normal environment. This is probably the significance of the increase shown by Pearse and Macpherson (1958) in the kidney of the potassium-deficient rat.

Pathological applications of the standard methods for acetylcholinesterases and pseudocholinesterases have been limited in number and restricted practically to the skin and to the central nervous system. Glial cells, and particularly the fibrous astrocytes, have been reported to contain a strong pseudocholinesterase (Koelle, 1954; Cavanagh, Thompson, and Webster, 1954), but Pepler and Pearse (1957) were unable to show this in human astrocytes or in astrocytomas. Both of the standard histochemical methods for acetylcholinesterases are sensitive, specific, and reliable, and their application to neuropathological problems has already produced useful results.

Among the many methods for glycosidases, that for β-glucuronidase has been most often applied to pathological problems. Recent improvements in one of the standard methods by Fishman and Baker (1956) should encourage its application in the fields of endocrinology and neoplasia. Once again, however, the results may have to be interpreted in the absence of knowledge as to the exact part played by the enzyme. According to Monis and Rutenberg (1956), the demonstration of β-glucuronidase activity may be useful for the

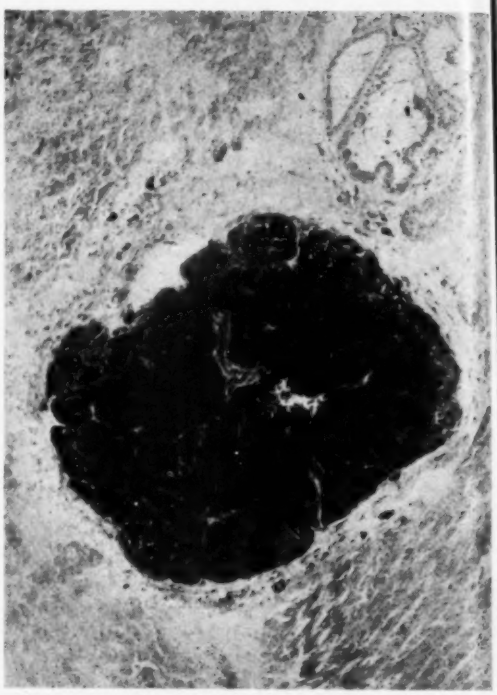
differentiation of epithelial and mesodermal tumours, since the latter do not possess the enzyme.

Diagnostic uses of enzyme histochemistry, as applied to pathological histology, are at present not numerous. This is hardly surprising, however, in view of the short period during which most of the applicable methods have been in existence. Further developments can only take place when the methods become more or less routine ones in histopathological laboratories. For this purpose the training of histochemical technicians is essential.

Another group of enzymes which has recently been brought into the histochemical orbit is the intracellular peptidases, particularly leucine aminopeptidase (Burstone and Folk, 1956; Nachlas, Crawford, and Seligman, 1957). Interest has centred on the production of the enzyme by invading tumours (Burstone, 1956) and on the rich source of the enzyme found in the stroma of such tumours. Sylvén and Malmgren (1955), using biochemical methods, found that the peripheral portions of invasive tumours contained higher cathepsin and dipeptidase activities than the older, central areas, and Braun-Falco (1957) has suggested that the production of aminopeptidase may be connected with invasiveness.

The activity of leucine aminopeptidase in the production of parathyroid hormone has been considered by Pearse and Tremblay (1958), who have demonstrated a presumably direct relationship between the two. The uniform distribution of the enzyme in normal rat parathyroid gland is shown in Fig. 1. The specificity of the histochemical method for leucine aminopeptidase has not been proved, so that, although it is interesting to speculate that either the parathyroid hormone, or its carrier protein, may have a terminal leucyl group, this point cannot be settled by histochemical methods. It remains certain that aminopeptidase activity is directly related to the production of parathyroid hormone.

The non-specific esterase methods have been widely used in applied histochemistry, but there has unfortunately been little effort to distinguish the various types of esterase activity on the basis of findings and classifications recorded in the biochemical literature. It is possible, following the work of Holt (Holt, 1952; Holt and Withers, 1952, 1958), to show with considerable accuracy and reasonably quantitatively the position in the cell of a number of non-specific esterases, using halogen-substituted indoxyl acetates as substrates. The evaluation of changes observed in the level



All photomicrographs are from 4 or 8 μ fresh frozen cold microtome (cryostat) sections.

FIG. 1.—Normal rat parathyroid gland (embedded in liver). Shows high activity of leucine aminopeptidase. L-leucyl-β-naphthylamide. × 120.

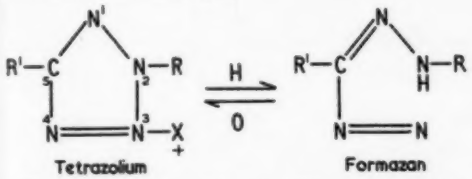
and distribution of these enzymes is difficult, however, since their exact function is poorly understood. The accurate degree of localization which these methods afford, however, and the possibility of determining small intracellular changes in function, make it essential to continue with their application on a much wider scale. Now that the substrates are more easily available former objections to the indoxyl methods must vanish.

It is unfortunately true of the majority of enzymes for which histochemical methods are available that their functional significance is obscure. When we come to the dehydrogenases and diaphorases, however, we observe a significant difference. This is because biochemical knowledge of the function of these enzymes, within and outside the tricarboxylic acid cycle, is much more advanced than it is in the case of the other enzyme systems. Thus, if we can localize the dehydrogenases histochemically by methods in which the amount of colour developed is propor-

tional to the rate of enzyme activity, we are in a position, as never before, to draw level with the biochemist in revealing the mechanisms by which the cell carries out its function in health and disease. We can then say something, at least, about the organization of events within the cell. I propose, therefore, to conclude this paper by considering briefly the current histochemical position in respect of the dehydrogenase systems, since I believe that the most promising developments in modern applied enzyme histochemistry are to be found in this field. Only one other field promises such advances. This is the division of immunohistology, based on the original fluorescent antibody methods of Coons and his associates (Coons, Creech, and Jones, 1941 ; Coons, Creech, Jones, and Berliner, 1942).

Dehydrogenase Histochemistry

Histochemical methods for the dehydrogenases are all derived from the original method of Seligman and Rutenburg (1951), which was derived in its turn from the successful use by Rutenburg, Gofstein, and Seligman (1950) of the so-called blue tetrazolium salt for the demonstration of dehydrogenase activity in extracts and homogenates. The principle depends on the acceptance of electrons by the colourless, soluble tetrazolium salt and its reduction to a coloured, insoluble, formazan dye.

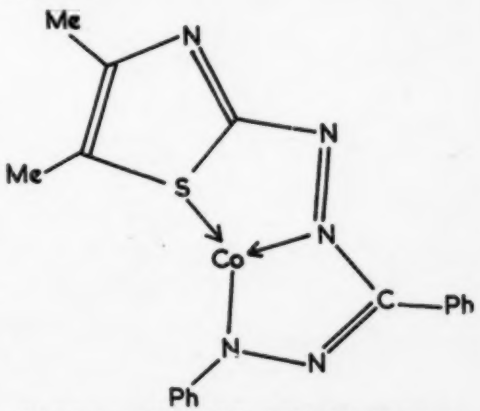


The reaction is not reversible in biological systems, and the amount of formazan deposited can be related directly to the amount of enzyme activity (Defendi and Pearson, 1955 ; Glick and Nayyar, 1956). Developments which have taken place since 1951 have brought the dehydrogenase methods into the forefront of enzyme histochemistry. A series of papers by Farber and his associates (Farber, Sternberg, and Dunlap, 1956a and b ; Sternberg, Farber, and Dunlap, 1956 ; Farber and Louviere, 1956) set forth the authors' hypothesis that attempts to localize specific dehydrogenases by tetrazolium methods result only in the demonstration of one or other of the two pyridine-nucleotide-linked diaphorases. These flavoprotein enzymes transfer hydrogen from the substrate to the acceptor, in this case a dye or tetrazolium salt. The succinic dehydrogenase system forms an exception since it contains its own built-in flavoprotein and does not require the intervention of a diaphorase in transferring electrons from succinate to tetrazolium salts.

If Farber's hypothesis is true then the information which can be derived from the application of the tetrazolium methods will be severely limited in scope. If, on the other hand, the demonstration of specific dehydrogenases is shown to be possible the whole field is transformed. The significance of such a possibility in applied histochemistry can hardly be overestimated.

An outstanding drawback of all the tetrazolium methods in use up to 1957 was the crystalline nature of the formazan reaction product. The most commonly used tetrazolium salts (neotetrazolium, blue tetrazolium) both gave rise to two products, a red one soluble entirely in tissue lipids and a blue or purple one which formed thick needle-shaped crystals. It was thus impossible to obtain enzyme localization at a cytological level. These objections have recently been overcome, in two different ways, by the development of new tetrazolium salts.

The first of these, nitro-blue tetrazolium (Nitro-BT), was produced by Tsou, Cheng, Nachlas, and Seligman (1956) and applied by Nachlas, Tsou, de Souza, Cheng, and Seligman (1957) to the intracellular localization of succinic dehydrogenase. Nitro-BT is capable of accurate localization because its formazan has a strong binding capacity for protein (substantivity), and it is thus able neither to diffuse far from its site of origin nor to crystallize.



Cobalt formazan from 3-(4,5-dimethylthiazolyl-2-)2,5-diphenyl tetrazolium bromide (Beyer and Pyl, 1954).

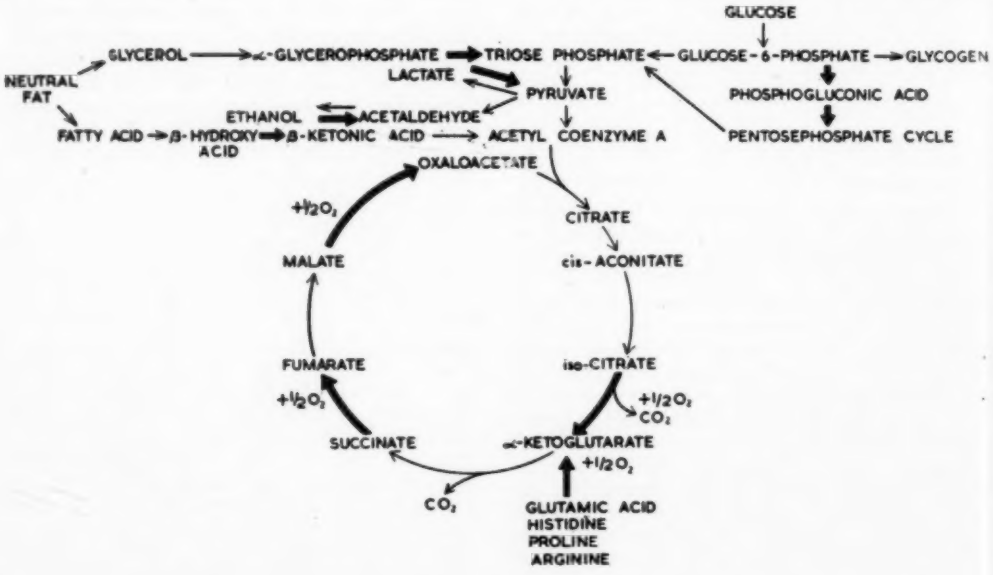
An entirely different principle is involved in the second method (Pearse, 1957) in which the formazan reduction product of a thiazol-substituted monotetrazolium salt (Beyer and Pyl, 1954) is captured *in situ* by chelation with a metal ion.

The usual metals are cobalt, nickel, and copper, in that order of preference. Both of the new methods are capable of localizing enzyme activity at the mitochondrial level (Figs. 2 and 3). In both cases the deposits of diformazan or cobalt-monoformazan are present in the mitochondria in the form of dots, measuring 0.25 to 0.35 μ in diameter (Pearse and Scarpelli, 1958). These are much more easily seen with the cobalt-formazan technique. An average mitochondrion 3 μ long may contain four formazan dots; the greatest number recorded in a single mitochondrion is six.

The view that tetrazolium methods can demonstrate only the two diaphorases and not the specific dehydrogenases with which they are linked has been shown to be erroneous by Nachlas, Walker, and Seligman (1958) and also by Hess, Scarpelli, and Pearse (1958). It is now possible, in fact, to demonstrate cytochemically the presence of nine of the pyridine-nucleotide-linked dehydrogenases, and these, together with succinic dehydrogenase, are shown in the diagram below by thick arrows. Stages indicated by thin arrows cannot at present be shown cytochemically.

Although homogenization and ultracentrifugation techniques have indicated that certain of the oxidative enzymes are intramitochondrial, others are consistently shown to be present mainly or entirely in other fractions. β-Hydroxybutyrate dehydrogenase, for example, is regarded as wholly mitochondrial, while the DPN-linked *iso*-citrate dehydrogenase appears partly in the mitochondrial fraction and partly elsewhere. Glucose-6-phosphate dehydrogenase is found exclusively in the supernatant. The newer cytochemical methods suggest that these three enzymes are all intramitochondrial (see Figs. 2, 4, and 5). The two diaphorases show a similar intramitochondrial pattern of localization (Fig. 6) and the biochemically recorded microsomal TPNH-diaphorase cannot be demonstrated cytochemically by the cobalt-formazan technique (Scarpelli, Hess, and Pearse, 1958). Virchow, whose colostrum corpuscle was a "coherent globule" resulting from the fatty degeneration of an epithelial cell, would perhaps have enjoyed the macrophage from the breast of a lactating rat, shown in Fig. 4. This contains a "modern" colostrum corpuscle surrounded by short mitochondria typically rich in glucose-6-phosphate dehydrogenase.

The ability to demonstrate effectively so many of the mechanisms of intracellular oxidation is obviously of great significance in relation to bio-



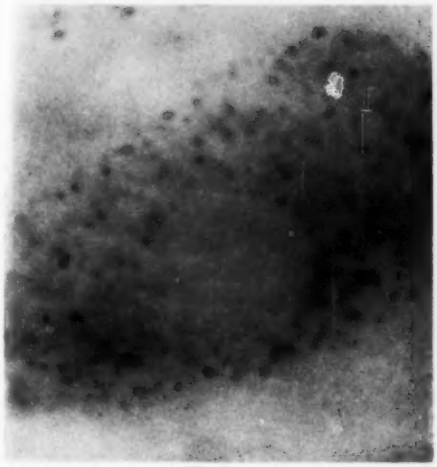


FIG. 2

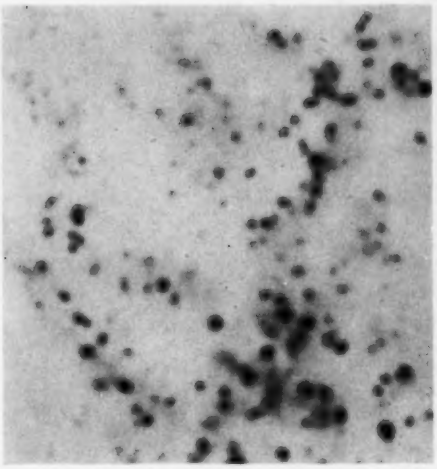


FIG. 3

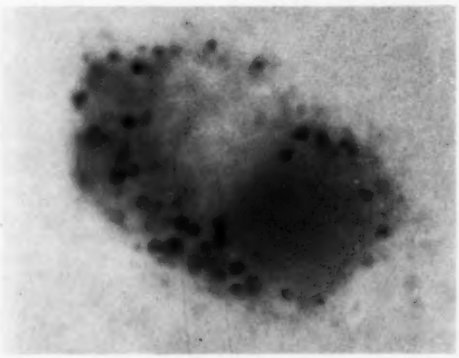


FIG. 4

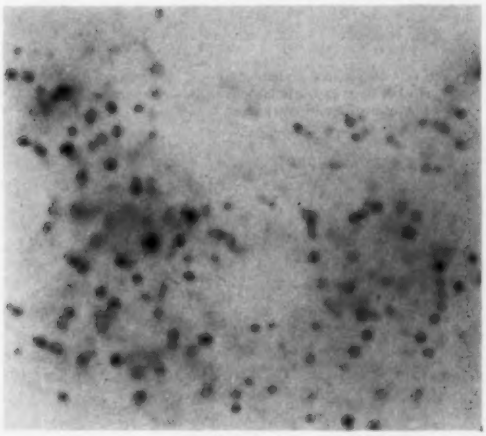


FIG. 5

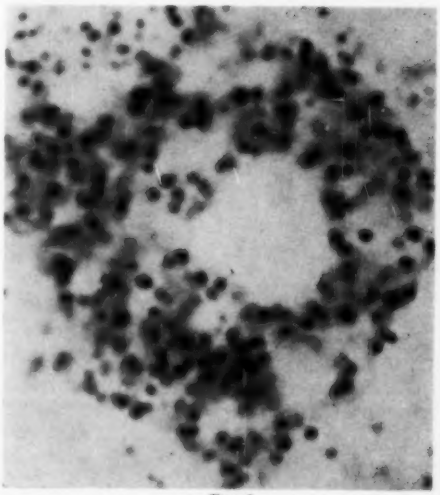


FIG. 6

FIG. 2.—Rat stomach. A single parietal cell. Shows distribution of DPN-linked *iso*citrate dehydrogenase activity. Nitro-BT. × 1,840.

FIG. 3.—Rat kidney. Proximal convoluted tubule. Shows succinic dehydrogenase activity in the mitochondria. Cobalt-tetrazolium method. × 4,300.

FIG. 4.—Rat breast (lactating). A single macrophage containing (lower right) a colostrum corpuscle. Shows glucose-6-phosphate dehydrogenase activity in the mitochondria. Cobalt-tetrazolium method. × 4,300.

FIG. 5.—Rat kidney. Portions of a number of tubule cells. Shows β-hydroxybutyrate dehydrogenase activity. At lower right appears a single mitochondrion containing five formazan "dots." Cobalt-tetrazolium method. × 4,300.

FIG. 6.—Rat salivary gland. DPNH-diaphorase activity in a single duct cell. Cobalt-tetrazolium method. × 4,300.

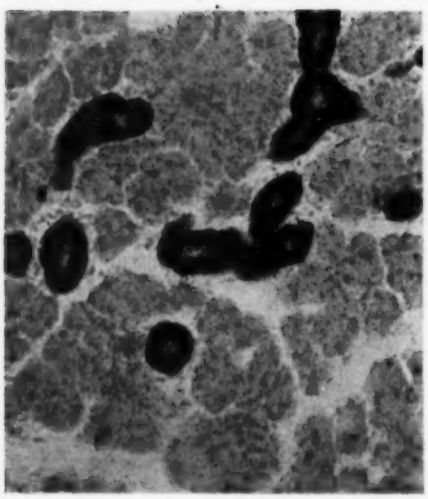


PLATE Ia.—Rat salivary gland. Shows high activity in duct lining cells but poor intracellular localization. Succinic dehydrogenase. Blue tetrazolium, methyl green, × 200.

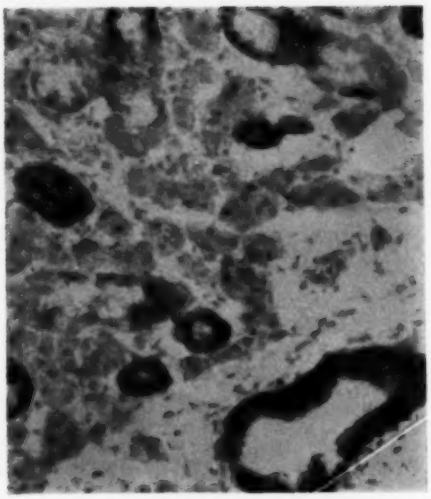


PLATE Ib.—Rat salivary gland. Shows great improvement in intracellular localization afforded by the use of a tetrazolium salt having a substantive formazan. Succinic dehydrogenase. Nitro-BT, methyl green, × 200.

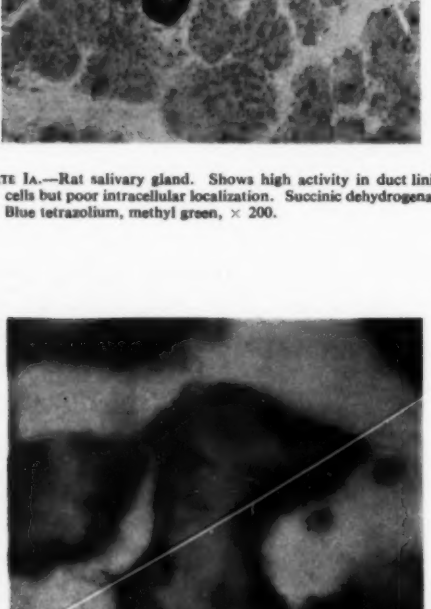


PLATE Ic.—Rat kidney. Distal convoluted tubules. Similarly improved localization provided by an efficient capture reaction. Succinic dehydrogenase. Cobalt-tetrazolium, methyl green, × 700.

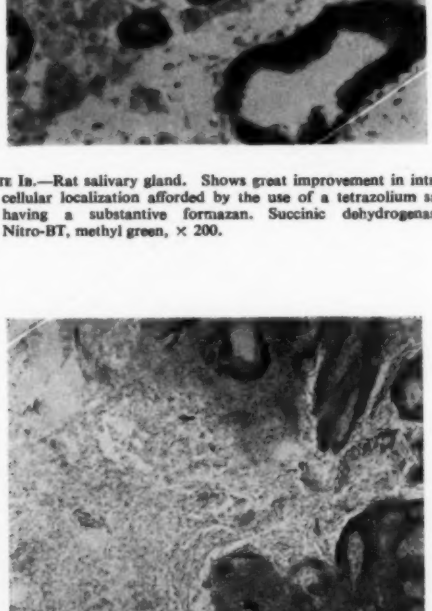


PLATE Id.—Mouse cervix. Methylcholanthrene-induced squamous cell carcinoma. Growing edge of the tumour. Solochrome cyanine RS. × 124.

logical and pathological problems. It becomes possible to follow small alterations in the level of the preferential oxidation pathway in single cells and to detect changes from one predominant pathway to another. This cannot be achieved by biochemical methods except in the case of homogeneous tissues. Even then, as Schneider (1956) has emphasized, the result is the average of the contribution of a number of different cell types, and of cells of a single type with widely differing enzyme contents. Speaking of the standard biochemical homogenization techniques, with which he and others have made so considerable a contribution to our knowledge of intracellular oxidations, Novikoff (1956) says, "It is well recognized that the activity of a given fraction obtained by differential centrifugation is an average figure." He goes further and says, "Even if it were possible to free the mitochondrial fraction, for example, of all contamination by other cell particulates, enzymic differences existing among diverse mitochondria within a cell, and among different cell types, would still be merged into the average." "This limitation," he says, "is particularly troublesome when the organism is subjected to altered conditions which may shift the relative proportion of different mitochondria within a cell or of different cell types in the organ." Dehydrogenase cytochemistry is free from these limitations though subject to certain others. Absolute quantitation of enzyme activity, for instance, cannot at present be achieved. Nevertheless, the sensitivity of the histochemical methods is such that β-hydroxybutyrate activity can be demonstrated in the particles of Keilin-Hartree preparations although such activity is not detectable by the usual manometric (Warburg) methods.

Application of the newer histochemical methods for dehydrogenases and diaphorases has already shown clearly that the mitochondrial population is not homogeneous from cell to cell, or even within a single cell, and that this lack of homogeneity occurs not only with reference to content of specific enzymes but also with reference to physical states, such as the permeability of the mitochondrial membrane and the sensitivity of the organelle to environmental influences.

We can now show, therefore, not only the differences between one tissue and another and between one cell and another, but also between one mitochondrion and its neighbour.

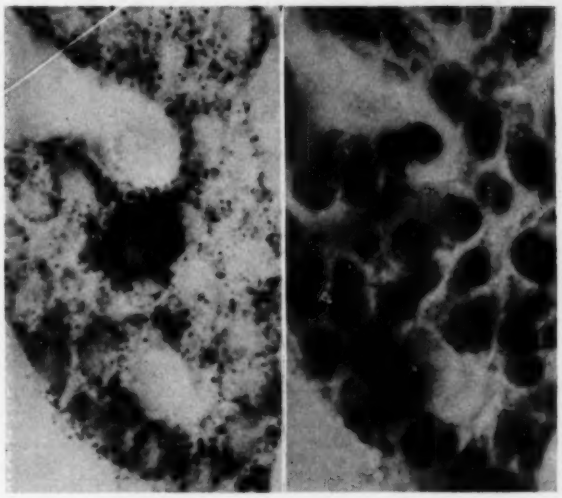


FIG. 7 FIG. 8

FIGS. 7 and 8.—Part of parathyroid gland of monkey (*Macaca irus*). Fig. 7 shows a high activity of DPNH-diaphorase in a single oxyphil cell. The same cell appears in Fig. 8, stained with haematoxylin and eosin. ×900.

Applied Histochemistry of the Dehydrogenases and Diaphorases

It is unlikely that biochemical studies on the DPN-linked dehydrogenases of the parathyroid gland have yet been carried out. If they have, they will have indicated a moderate level of activity little influenced by physiological changes in function. Studies on succinic dehydrogenase will show a similarly moderate and constant level of activity. Histochemical studies, on the other hand (Tremblay and Pearse, 1959), reveal that DPNH-diaphorase, and five of the histochemically demonstrable dehydrogenases which require this enzyme, are concentrated in the oxyphil cells. The latter are not present in the majority of species. In the monkey, however, they are well developed and the glands can be obtained in the fresh state necessary for the successful application of the methods. Figs. 7 and 8 show a single oxyphil cell, first with its content of intramitochondrial DPNH-diaphorase, and subsequently, after removal of the metal formazan, by staining with haematoxylin and eosin.

A similar concentration of DPNH-diaphorase is shown in Fig. 9, in a symplastic giant cell in a sarcoma produced experimentally in a mouse by

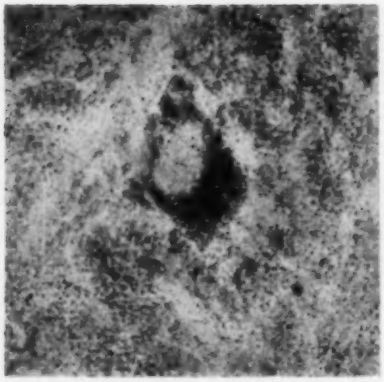


FIG. 9.—Mouse tissue. A methylcholanthrene-induced sarcoma. Shows a high activity of DPNH-diaphorase in a so-called symplastic giant cell. Low activity in the other tumour cells. Cobalt-tetrazolium method, carmalum. × 640.

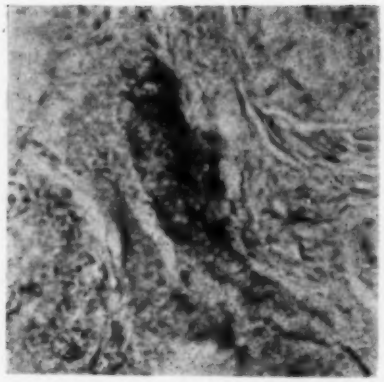


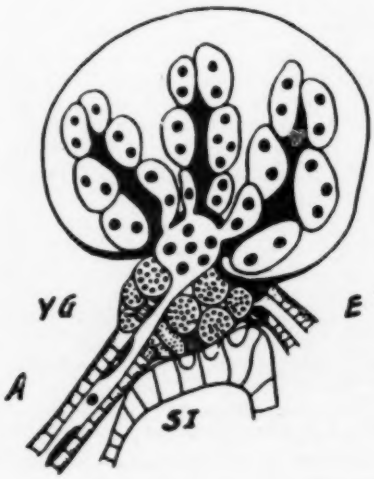
FIG. 10.—Rat kidney. Normal macula densa shows glucose-6-phosphatase activity. Cobalt-tetrazolium method, carmalum. × 1,000.

implantation of methylcholanthrene. In both cases it is tempting to assume that this intense enzyme activity is related to increased capacity for oxidation, and that this in its turn is related to the synthesis of products as yet unknown.

Histochemical studies have also shown that there is a special localization of 6-phosphogluconate dehydrogenase in the macula densa of the normal rat kidney (Nachlas *et al.*, 1958), and this has been confirmed by Hess and Pearse (1959) in the case of this enzyme and also for glucose-6-phosphate dehydrogenase. In experimental hypertension in the rat, induced by means of a Goldblatt clamp, the clamped kidney shows no activity of glucose-6-phosphate dehydrogenase except in the macula densa and in the arterial and arteriolar walls. In the unclamped kidney the amount of enzyme in the macula densa falls to low levels. The appearance of the normal macula densa in the rat is shown in Fig. 10, where the activity of the glucose-6-phosphate dehydrogenase in the mitochondria of the five or six cells concerned is evident. If biochemical studies were carried out a low level of enzyme activity would be recorded, and it is possible that in the early stages no difference could be shown between the clamped and unclamped kidneys. Hess and Pearse have shown that, as far as the macula is concerned, the level of glucose-6-phosphate dehydrogenase rises in the clamped kidney as the development of hypertension proceeds. Changes in the rabbit macula densa in hypertension were recorded by Goormaghtigh (1939), who described swelling and vacuolation of the cells (marked SI in the diagram below, which is reproduced from his paper), but no evidence of increased activity has hitherto been recorded. The juxtaglomerular apparatus (YG) is ultimately involved, in long-standing hypertension, in the general arteriolar increase in glucose-6-phosphate dehydrogenase.

Further Examples in Applied Dehydrogenase Histochemistry

The inhomogeneity of cells in a single type is well shown by reference to various types of tumours. Plate ID shows the advancing edge of a squamous carcinoma of the mouse cervix, pro-



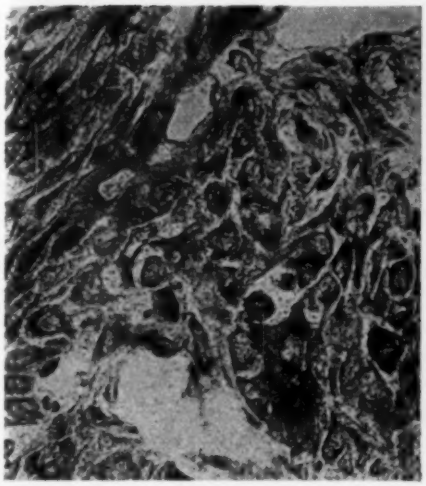


FIG. 11

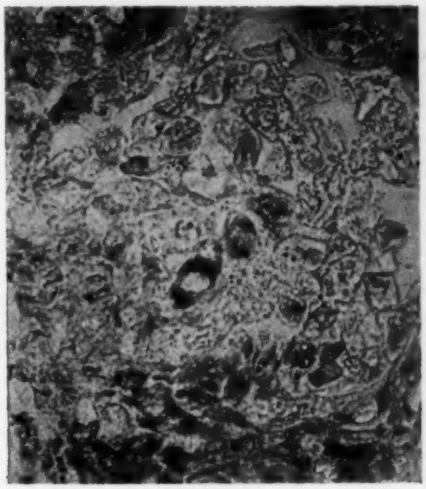


FIG. 12

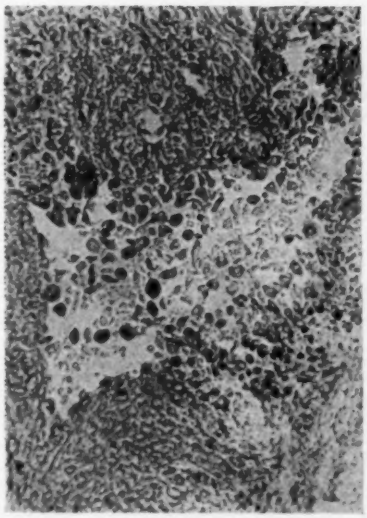


FIG. 13

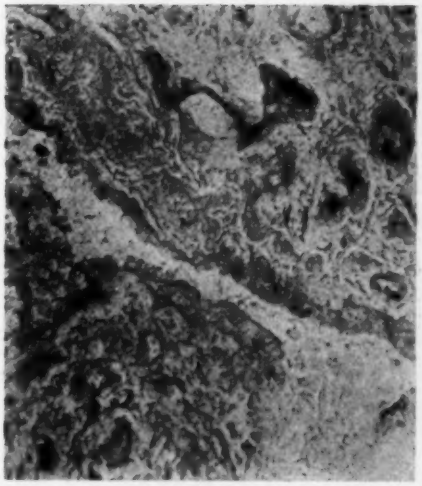


FIG. 14

FIG. 11.—As Plate ID. DPNH-diaphorase reaction. Shows wide variation in level of activity in healthy cells. Cobalt-tetrazolium method. × 490.

FIG. 12.—As Fig. 11, but shows a region of keratinization. Low enzyme activity in most cells. Cobalt-tetrazolium method. × 475.

FIG. 13.—As Fig. 12. Shows very high activity of glutamate dehydrogenase in cells which are about to keratinize. Nitro-BT. × 630.

FIG. 14.—As Plate ID. Growing edge of tumour. Shows high activity of glucose-6-phosphate dehydrogenase in the advancing cells. Cobalt-tetrazolium method. × 630.

duced experimentally by implantation of methylcholanthrene (Scarpelli and von Haam, 1957), which has been stained in a conventional manner. In the next illustration (Fig. 11) a similar area has been shown to demonstrate the localization of DPNH-diaphorase in the mitochondria. Histologically all the cells present are healthy and viable, and without distinguishing features. The enzyme method shows, however, that some have little activity and others, such as the cell in the lower right-hand part of the picture, are intensely active. This difference in activity of DPNH-diaphorase is not unnaturally much greater in the keratinizing areas of the tumour (Fig. 12) where the fully keratinized cells contain little or no enzyme. There is an interesting change observable in cells which are just about to keratinize (Fig. 13), where a pronounced rise in glutamic dehydrogenase is seen. There is no parallel rise in any of the other dehydrogenases. An entirely different picture is seen in the growing edges of the tumour (Fig. 14). Here, there is a relatively enormous rise in glucose-6-phosphate dehydrogenase activity and this is closely followed by a rise in ribonucleic acid levels within the cells.

Clearly these results are significant and we have to ask ourselves, or the biochemists, what they mean. In Table I the predominant oxidative mechanism associated with a given enzyme system is recorded.

TABLE I

SUGGESTED SIGNIFICANCE OF DEHYDROGENASE ACTIVITIES

Substrate Utilized	Co-enzyme	Mechanism Involved or Significance
Succinate	None	Citric acid cycle
β-OH butyrate ..	Co I	Fatty acid oxidation→*active* acetyl synthesis
Glutamate	Co I	Synthesis or breakdown of glutamate Urea or creatine formation
Malate	Co I	Citric acid cycle
*Iso*citrate	Co I or Co II	{ α-Ketoglutarate utilization citric acid cycle
Glucose-6-phosphate	Co II	Metabolism via oxidative (hexose monophosphate) shunt synthesis of { A.T.P. Nucleotides
α-Glycerophosphate	Co I	Glycolysis (early stage)
Alcohol	Co I	Glycolysis
Lactate	Co I	Glycolysis (later stage)
DPNH	Diaphorase	Total activity of Co-I-linked dehydrogenase systems α-Lipoic acid shunt
TPNH	Diaphorase	Total activity of Co-II-linked dehydrogenase systems

Most of the information in the table is derived from biochemical studies and obviously requires amplification. Such amplification is beyond the scope of this article.

Indications of the Physical State of Mitochondria

The mitochondria fresh frozen sections are altered in some respects by the freezing and thawing involved. They can no longer carry out oxidative phosphorylation, for example. If steps are taken in histochemical practice to preserve the integrity of the mitochondria as far as possible, by avoiding heating or drying and by their protection during incubation with the use of hypertonic media (7.5% polyvinyl pyrrolidone), the appearances with the latest tetrazolium methods are those shown in Figs. 2–6. The mitochondria are still intact, as can readily be ascertained by inspection with the phase microscope. If for any reason a mitochondrion is damaged before incubation, so that the membrane is abnormally permeable, the first result is that the reaction rate increases and more formazan per unit time is deposited. The appearances of such mitochondria are recorded in the right-hand tubule of the kidney section shown in Fig. 15. The middle tubule contains essentially normal mitochondria. If the damage is so severe that the mitochondrial membranes are ruptured the appearances in the section approach those seen in the left-hand tubule in Fig. 15.

If an osmotic load is put upon the mitochondria, by pre-incubating the fresh frozen sections in hypotonic or otherwise harmful media, those which are abnormally sensitive are observed to respond sooner and more violently than the normal ones. We thus have a tool which is capable of showing very early changes in the physiological state of the mitochondria, and we can therefore indicate pathological changes in a variety of conditions a long time before any change can be seen by conventional methods.

In Fig. 16 is shown the TPNH-diaphorase reaction in the juxtamedullary tubules of the renal cortex of a rat maintained for 36 hours on a diet deficient in magnesium. Swollen (damaged) mitochondria, sometimes only one per cell, are visible. The renal tubules in other areas show absolutely no abnormality. The change is more easily seen at higher magnification, as in Fig. 17 (succinic dehydrogenase) and more easily still in Fig. 18, which shows the same tissue pre-incubated for 30 minutes in a hypotonic medium. Such treatment produces no response from the normal mitochondria of these tubules (though it may from the more sensitive normal mitochondria of other organs such as the parietal cell of the rat stomach shown in Figs. 19 and 20). It is thus possible to calibrate every type of mitochondrion in every tissue and to apply to it just that load which will

FIG. 15.—Rat kidney. Reaction of mitochondria to osmotic injury. This chance observation in a single section shows, left, disrupted mitochondria; right, swollen (damaged) mitochondria containing more formazan than the normal mitochondria of the central tubule. Succinic dehydrogenase. Cobalt-tetrazolium method. × 850.

FIG. 16.—Rat kidney. Juxtamedullary region of the cortex. Three days Mg deficiency. Shows early change in mitochondria (swelling and increased reaction rate). TPNH-diaphorase. Cobalt-tetrazolium method. × 750.

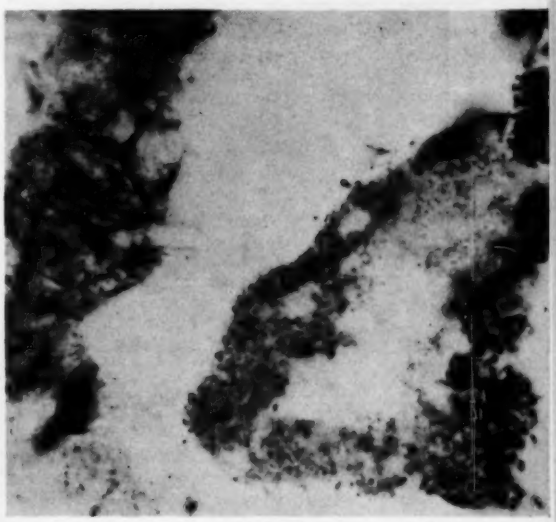


FIG. 15

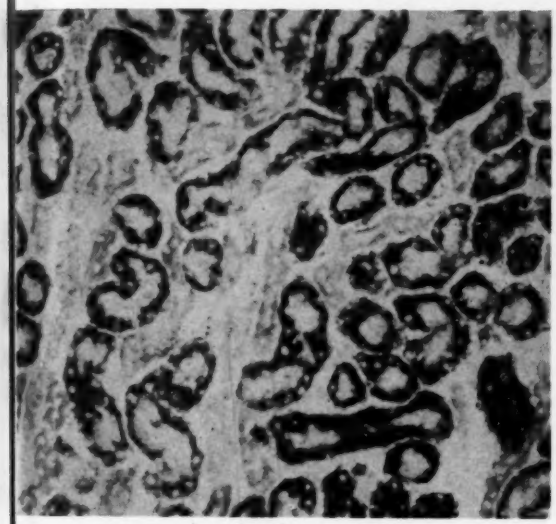


FIG. 16

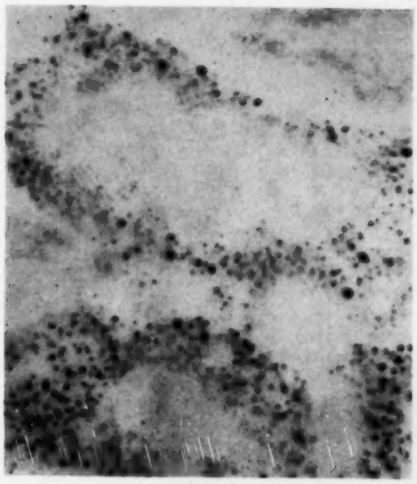


FIG. 18

FIG. 17

FIG. 17.—Rat kidney. Succinic dehydrogenase reaction after three days Mg deficiency. Shows early damage to mitochondria in tubule cells of the juxtamedullary region. Cobalt-tetrazolium method. × 900.

FIG. 18.—As Fig. 17, but section pre-incubated in hypotonic medium before succinic dehydrogenase reaction. Abnormal mitochondria show larger, darker formazan deposits. × 900.

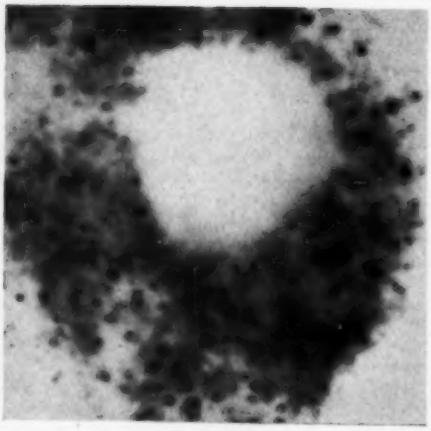


FIG. 19

FIG. 20

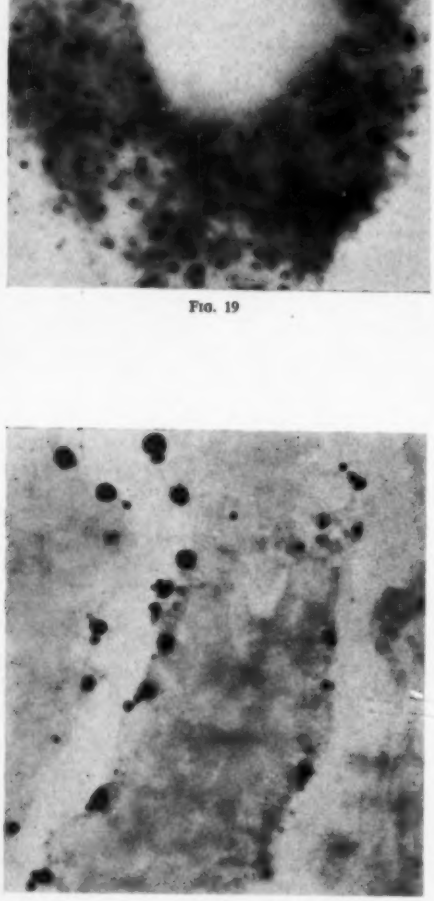


FIG. 21

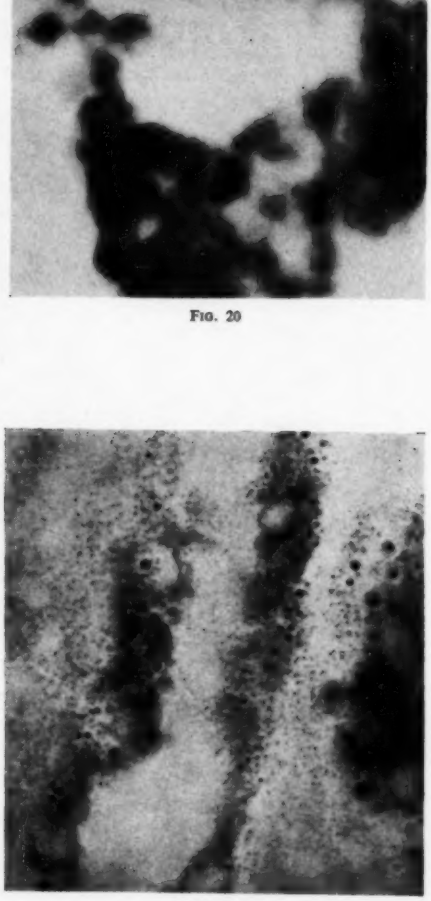


FIG. 22

FIG. 19.—Rat stomach. Parietal cell incubated for succinic dehydrogenase in a protective medium. Shows mitochondrial localization of enzyme. × 4,450.

FIG. 20.—As Fig. 19, but incubated in hypotonic medium. Individual mitochondria are entirely unrecognizable. × 4,450.

FIG. 21.—Rat kidney. Early stage of Mg deficiency (fifth day). Shows fatty degeneration. Oil red O. × 900.

FIG. 22.—Rat kidney. Similar stage to Fig. 21. Shows close association of early stages of fatty degeneration (clear halos) with mitochondrial damage or swelling. DPNH-diaphorase. × 900.

reveal the earliest change in sensitivity (permeability) of the membrane. The implications of such a method in experimental pathology need little amplification.

The gross changes in the kidney of magnesium deficiency, which include intracellular and interstitial calcification, become visible by standard techniques after a period of about nine days. They occur in precisely the area delineated by "mitochondrial assay" a matter of hours after the start of the experiment. Biochemical changes are noted on the fifth or sixth day, when the serum magnesium figures fall to low values. The "blindness" of the usual histological techniques needs no further emphasis.

A similarly early appreciation of the pathological changes has been observed in every condition to which the methods of "mitochondrial assay" have been applied. In poisoning with diphtheria toxin, for example, changes are visible in the sarcosomes of the cardiac muscle two hours after injection of the toxin. This compares with the seven days of the usual techniques. In renal calcification, induced by single doses of vitamin D, changes are similarly visible in a few hours in the region of the kidney where ultimately (nine to 15 days) massive calcification occurs. It is possible, incidentally, using sensitive fluorescent techniques for calcium (Pearse, 1959), to detect the deposition of calcium in the interstitial tissues and in some of the cells of the renal tubules by the fifth day of the experiment. Starvation is another condition which induces changes in animal tissues and these can likewise be appreciated by "mitochondrial assay" after only a few hours' deprivation of food (in the case of the rat). It is interesting to note that the fatty degeneration, described and studied by Dible and Gerrard (1938), and by other workers (Fig. 21), appears to be closely connected with the swelling of damaged mitochondria. This is seen in Fig. 22, where the swollen mitochondria which appear as fat droplets in the preparation stained with oil red are seen to contain small circular regions of formazan deposition. This is not the appearance of normal fat droplets, which are at first colourless, though they may subsequently develop autoxidation products which will reduce adsorbed tetrazolium salts if these are lipid soluble.

Conclusion

Enzyme histochemistry offers many things, first and foremost, an escape from misleading conceptions due to consideration of tissues as homogeneous. Dixon and Webb (1958), in their masterly work on "Enzymes," say that "it is by no means certain that even cells which are histologically of the same type have the same enzymic composition." In another passage they maintain that "unfortunately no information on the enzymic homogeneity of tissues is available." Enzyme histochemistry, on the other hand, demonstrates with certainty that histologically identical cells frequently have entirely different enzymic composition (examples have been given in this paper) and one can say that if "information on the enzymic homogeneity of tissues" is lacking there is already a considerable volume of information on their enzymic lack of homogeneity.

The diaphorase and dehydrogenase methods, a combination of DPNH-diaphorase and succinic dehydrogenase for example, provide a simple and reliable method for demonstrating the mitochondrial population of mammalian, invertebrate, and plant cells. Nobody should further consider for an instant the employment of older staining techniques for these organelles.

Although the true pattern of metabolic activity in cells will only become apparent in most cases when much more exploratory work has been done, one can say, with little exaggeration, that if a single mitochondrion in an organ, or at least in a section from that organ, is abnormal one can demonstrate this fact and determine to some extent the metabolic direction of this abnormality.

Such is the sensitivity of the techniques which I have described that there can be no doubt that probably more than 50% of all the studies in experimental pathology, carried out by whatever methods since the time of Virchow, can be repeated to-day with advantage and with the absolute certainty that new understanding of "the organization of cellular events" will result. A further application of these techniques can be foreseen in the field of experimental pharmacology where it will be possible to demonstrate the earliest effects of drugs upon cells in sections, smears, or tissue cultures, with obvious economy of effort.

I could wish that Rudolf Virchow were here to-day to describe for himself the possibilities for the extension of his new science of cellular pathology which I have tried, inadequately, to set forth in this article.

It should be evident, from the bibliography, that the results reported in this article are not "all my own work." I should nevertheless like to acknowledge the great help I have received from my colleagues, particularly Drs. Defendi, Grogg, Guerrero, Hess, Macpherson, Pepler, Scarpelli, and Tremblay.

The photomicrographs are the work of Mr. W. Brackenbury, and the drawings were done by Mr. F. G. Saunders.

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J. clin. Path. (1958), **11**, 535.

THE EFFECT OF REDUCED OXYGEN TENSION ON PLATELET FORMATION *IN VITRO*

BY

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It is now generally accepted that platelets are formed in the bone marrow from megakaryocytes. This suggestion was first clearly made by Wright (1906), who based his case on embryological and microscopical arguments as well as on considerations of comparative biology. The question was, however, settled when Thiéry and Bessis in 1956 produced their beautiful film showing conclusively that megakaryocytes throw out long processes which become fragmented to form platelets. This was confirmed by Izak, Nelken, and Gurevitch in 1957.

Examination in this laboratory of some hundreds of bone-marrow cultures from human beings and animals by tissue culture methods did not, however, in any case disclose activity in megakaryocytes (Pulvertaft and Humble, 1956). On two occasions only they were seen to rotate; on one spidery processes were seen reminiscent of the film by Thiéry and Bessis; in all other cases they remained spherical until, after a lapse of a few days, they disintegrated.

Nearly all the specimens examined in this series were, however, human; Thiéry and Bessis worked with the rat. More important is the fact that they worked with isolated megakaryocytes sealed between coverslip and microscope slide, whereas the specimens personally examined were mounted on agar in a special chamber.

It was found that when bone-marrow cultures were submitted to low oxygen tensions great activity was shown by megakaryocytes, including platelet formation.

Technique

The culture chamber used was similar to a blood-counting chamber, having a central pillar recessed by 2 mm., surrounded by a ditch which was normally half-filled with culture medium. The central pillar was coated with 2% agar, and the medium used was 10% human serum, 0.1% "difco" yeast extract, and 90% Hanks' balanced salt solution. Streptomycin and penicillin were added, 100 units per ml.

The marrow was collected into heparinized human serum, usually from the same patient. When this is poured into a dish and allowed to settle, small fragments are usually seen. These consist of fat and blood vessels with bone marrow, and the smallest possible fragments are placed on the agar. A coverslip is applied and sealed with beeswax. After half-filling the ditch with medium through a lateral aperture, later sealed, the preparation is incubated, coverslip downwards.

Agar repels cells, and glass attracts them; the marrow cells are therefore flattened on to the glass in a manner not seen unless agar is used. Motile cells move peripherally, and in 18 hours megakaryocytes are clearly seen as dense spheres. They congregate along blood vessels and are usually not obvious until migration is established (Figs. 1, 2, 3).

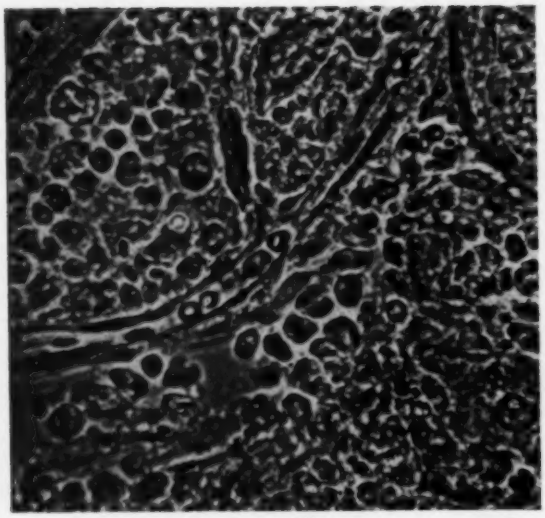
After 18 hours the culture medium is withdrawn, and the slide is placed in a desiccator with the lateral aperture unsealed. The desiccator is then evacuated with a mercury pump, and the air is replaced by a mixture of 95% nitrogen and 5% CO_2. Meanwhile culture medium is placed in shallow dishes, and all dissolved gas is removed by a mercury pump in a desiccator. When bubbling has ceased, 95% nitrogen and 5% CO_2 is introduced, and the desiccator is sealed for 18 hours, during which time the medium is saturated with this gas mixture. Five per cent. CO_2 is of course essential in order to maintain *p*H at 7.4. The chamber is then filled with this fluid, making sure that no bubble of air remains. It is sealed with beeswax.

The chamber is mounted on a phase-contrast microscope in an incubator box. Cinematographic records are taken by time-lapse.

Results

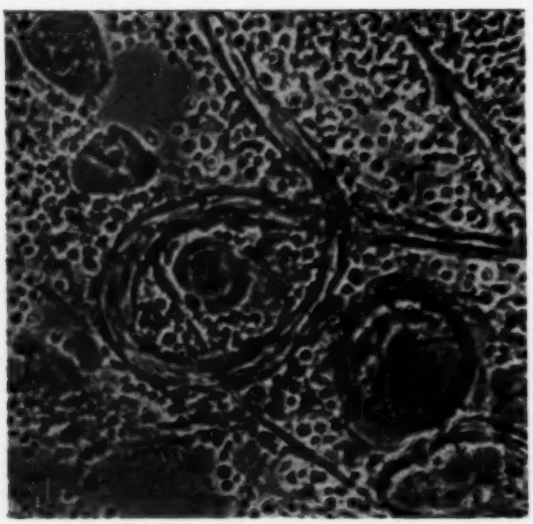
During the first five hours the cells normally motile continue to move, but the megakaryocytes remain spherical. After five hours motility of megakaryocytes is observed.

It must be appreciated that these cells are in various stages of maturity, and that only fully mature cells can form platelets. These can be recognized from their peculiar mottled texture, identical with the appearance of coherent platelets collected from the buffy coat of heparinized blood.



(a) × 560

(b) × 560



(c) × 240

(d) × 560

FIG. 1.—(a, b, c) Specimen of mouse bone marrow (normal) between glass surfaces, to show blood vessels and megakaryocytes.
(d) Peculiar capillary end body.

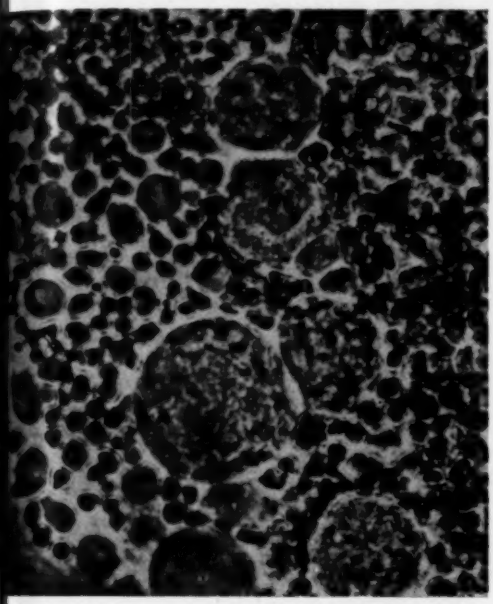


FIG. 2.—Megakaryocytes in medium saturated with air. × 560.

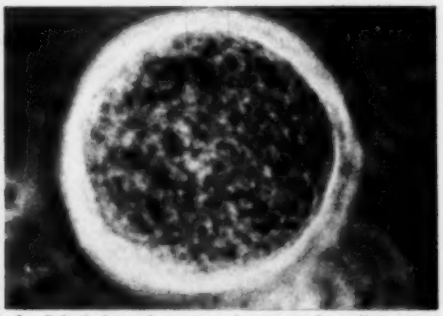


FIG. 3.—Spherical megakaryocyte. Appearance in medium saturated with air. × 840.

Four types of motility are observed. The first is rotation, in either direction, at a rate of one full rotation in three to four minutes. This continues for many hours, and rotating cells have not been observed to develop in any way. Although the nuclei of cells often rotate, I have never seen any other human cell rotating in nitrogen, in air, or in oxygen.

Secondly, megakaryocytes are seen to spread over the surface of the coverslip. This they do by sending out pseudopodia symmetrically all around, so that they cover a wide circular area, extending over many microscopical fields of a 1/12 lens. Such cells never develop further (Figs. 4 and 5).

They may also become sausage-shaped, and then become motile, progressing and elongating simultaneously (Fig. 6). Since they are at first spheres they make only point contact with the coverslip, and when this serpentine development sets in they are not adherent to the glass or agar, but progress in the fluid. Serpentine development may proceed to platelet formation.

Finally, true platelet formation develops by the extrusion of thick processes which elongate progressively and which eventually become fragmented. This process alone represents platelet formation, and only megakaryocytes which have the peculiar mottled appearance characteristic of maturity develop in this way (Figs. 7 and 8). Such cells in the early stages of platelet formation, while still spherical, are seen to be studded with small projections which make contact with the coverslip.

The very tenuous threads which are developed frequently are seen to expand and to contain nuclear fragments. The threads are readily broken up by motile cells, and it must be remembered that these "*in vitro*" experiments are unlike conditions in living marrow in that there is no circulation of blood; in life doubtless these threads are broken up by currents very quickly. In the slide preparations the threads often persist for 24 hours.

Those who do not examine living cells on a warm stage are apt to forget that the spherical forms seen in sections and stained films are not representative of living and active cells. The sphere is a defence mechanism, a form adopted when cells are chilled or otherwise insulted, and

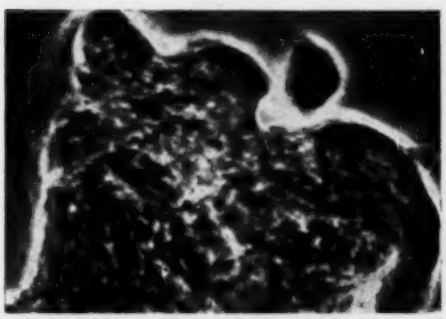


FIG. 4.—Spreading megakaryocyte, in nitrogenated medium. × 840.

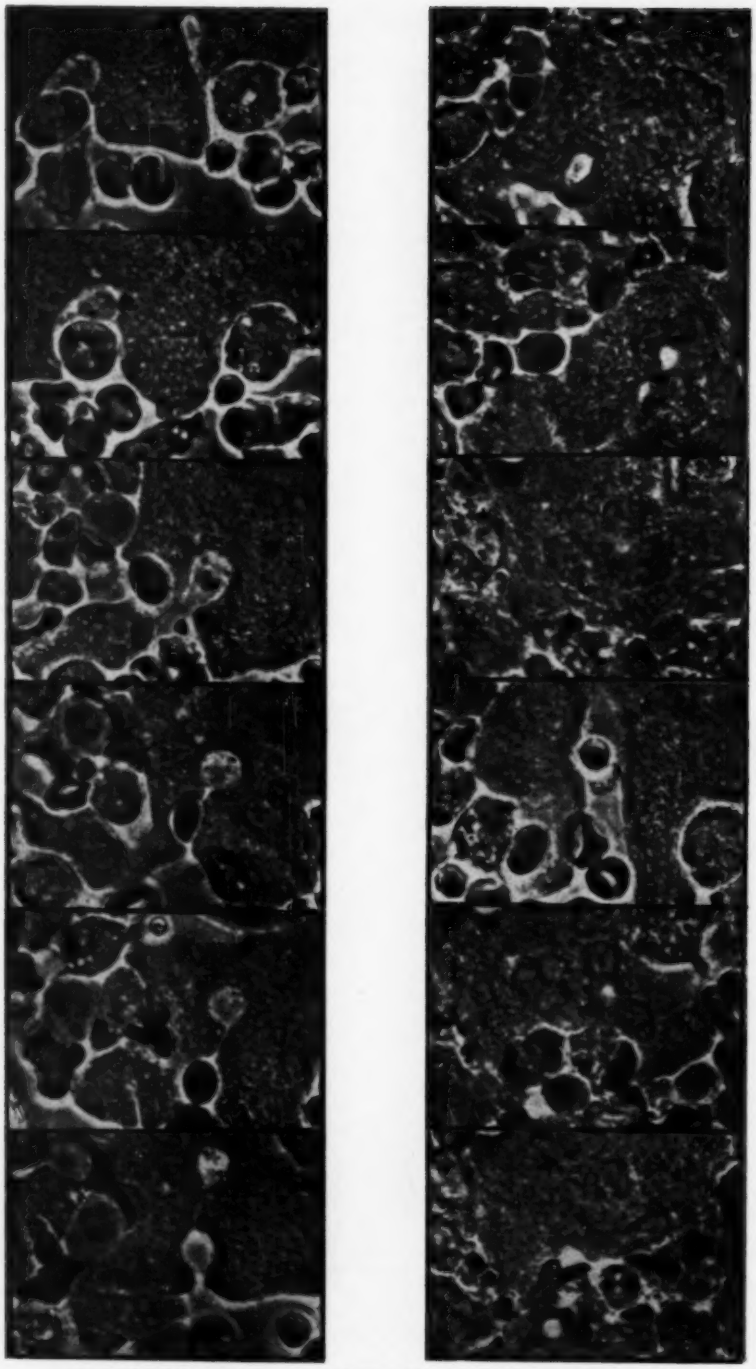


FIG. 5.—Spreading megakaryocyte, in nitrogenated medium. Sequence from film. × 840.

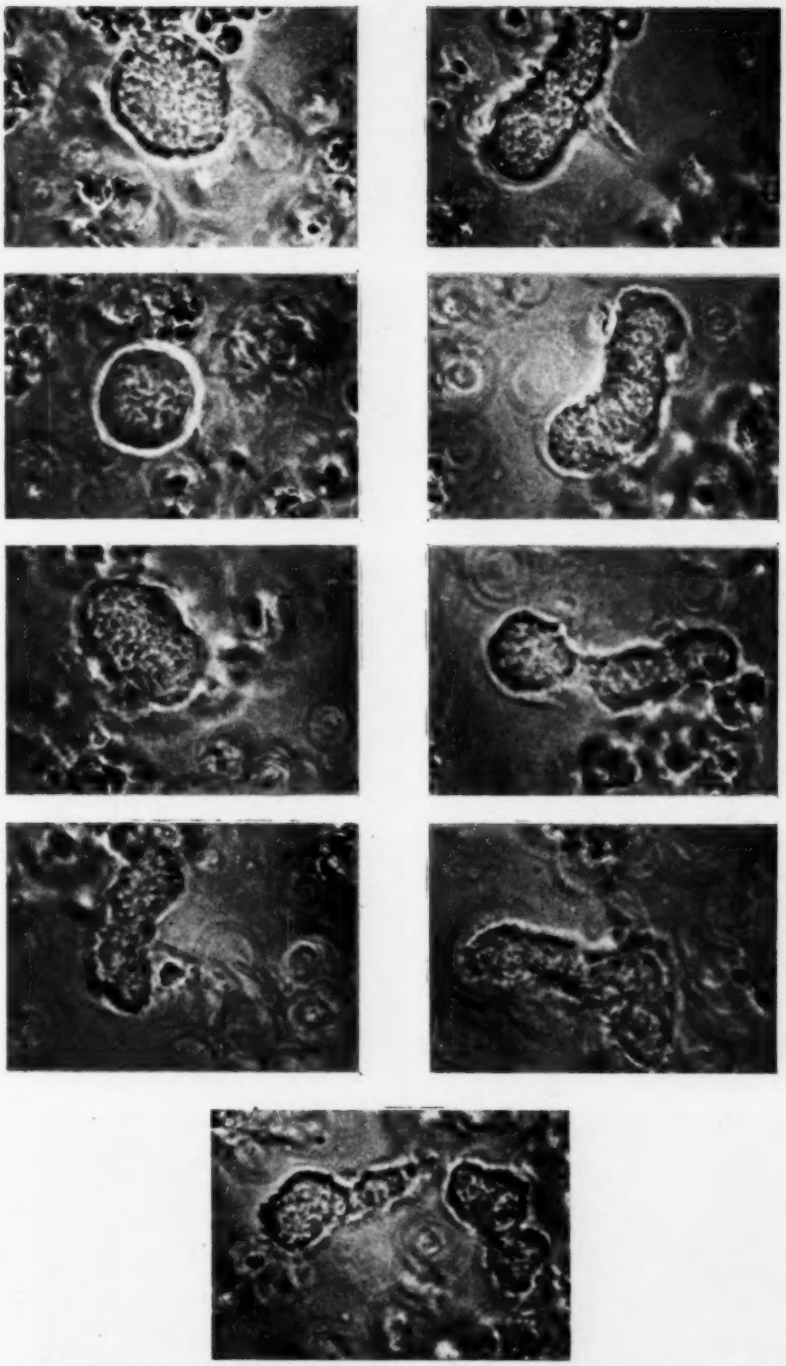


FIG. 6.—Elongating and progressing megakaryocyte, in nitrogenated medium. Sequence from film. × 390.

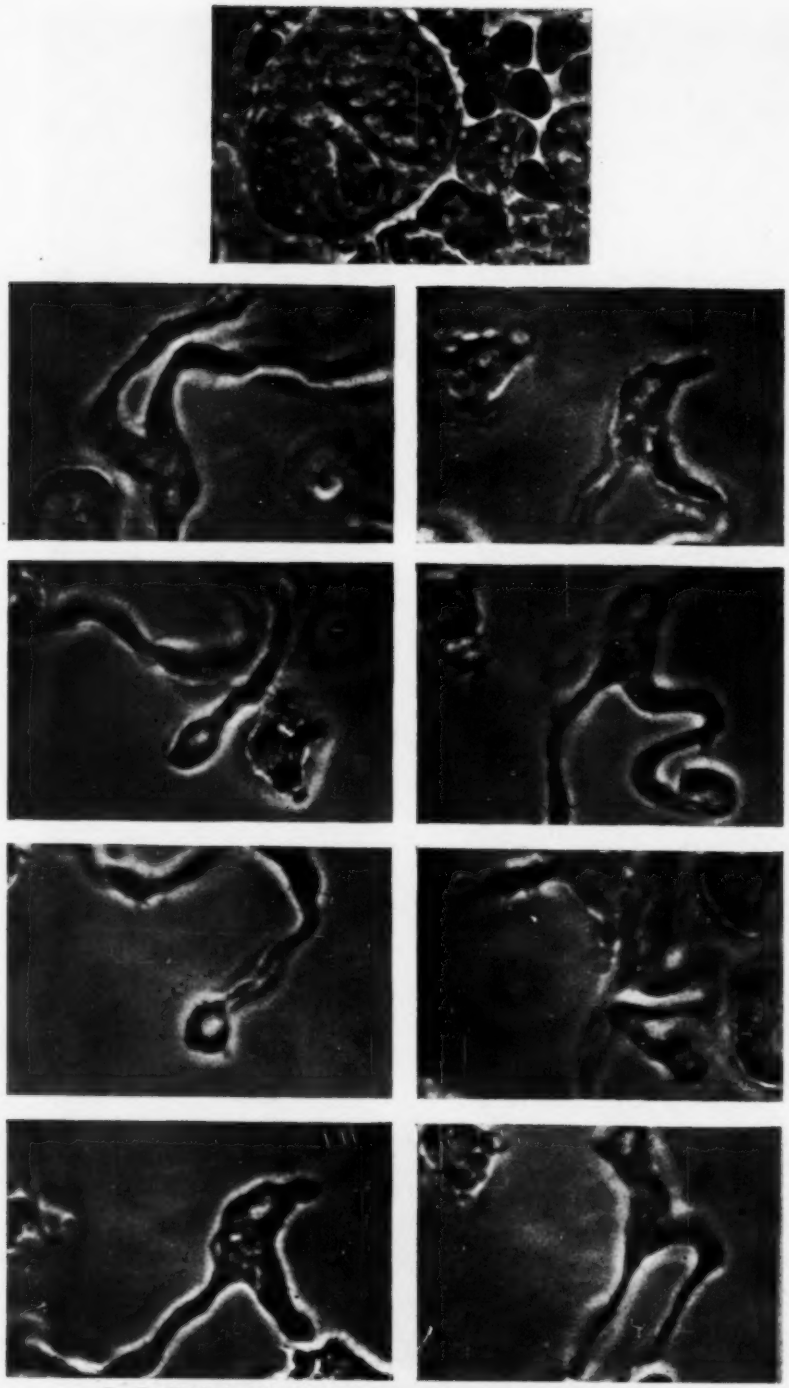


FIG. 7.—Platelet formation in nitrogenated medium. Sequence from film. × 840.

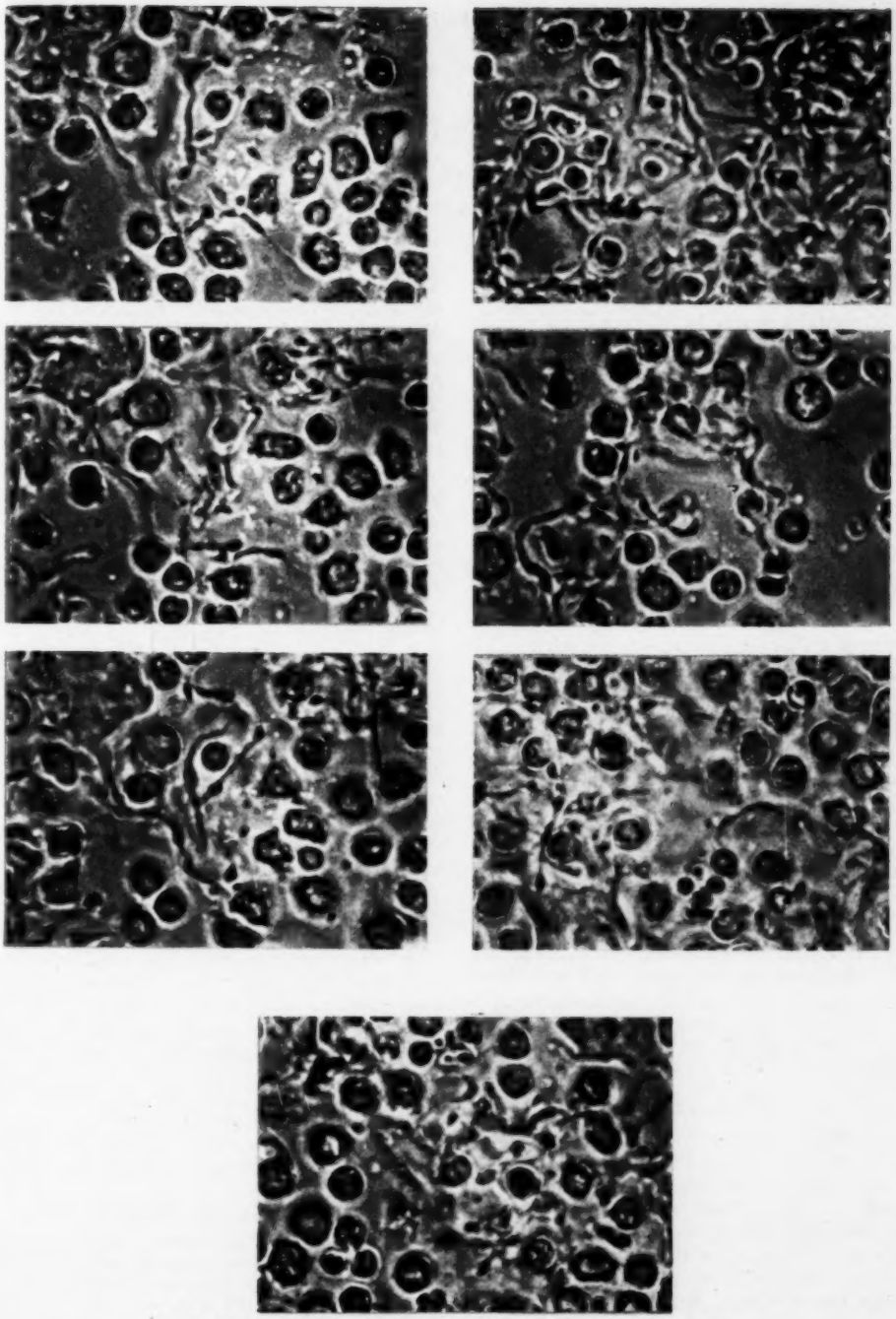


FIG. 8.—Platelet formation in nitrogenated medium. Sequence from film. × 390.

this is perhaps true of megakaryocytes as well as of polymorphs. We do not know from observation what the shape of a living megakaryocyte may be. They tend to collect around blood vessels, and therefore probably do not wander far. However, the number of controls in the present experiments is exceptionally large; about 2,500 bone-marrow preparations have been examined, in fluid saturated with air and 5% CO_2, without noting motility or any other activity in those cells, except the rotation twice observed. Almost invariably activity is seen in nitrogen after a lapse of approximately five hours.

It is not possible to say precisely how much oxygen is present in the preparations showing activity. The nitrogen used contained a maximum residuum of 0.1% oxygen, and not all the oxygen could have been removed from the agar cap by the vacuum.

The stimulus to megakaryocyte activity, including platelet formation, does, however, appear to be a sudden fall in oxygen tension, and this fits in with the known fact that platelets are increased following haemorrhage and in persons living at high altitudes. It is plausible to suggest that the reaction is a defence mechanism, considering the many ways in which platelets control haemorrhage.

The other forms of activity may be related to speed of megakaryocyte maturation. The larger the surface area of a cell the greater is its metabolic activity; oxygen deficiency may thus accelerate the development of these cells to the peculiar form which Bessis (1954) describes as thrombocytogenic. However, such maturation was not observed *in vitro*.

Summary

In the course of the examination of 2,500 slide cell cultures of human bone marrow, megakaryocytes remained spherical in all cases, and the only activity noted was rotation in two cases. When the oxygenated culture medium was replaced by one saturated with nitrogen and 5% CO_2, they showed activity after the lapse of five hours. The activity noted was (1) rotation, (2) elongation and progression, (3) wide spreading over a plane surface, (4) platelet formation.

All the bone-marrow samples and continuous advice were provided by Dr. J. G. Humble, consultant haematologist to Westminster Hospital, and technical help by Mr. J. A. Haynes, chief technician. Extracts from films were prepared by Miss A. Readman.

All the expenses were covered by a grant from the British Empire Cancer Campaign.

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THE ANTIBODY CONTENT OF SINGLE CELLS

BY

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Quantitative work on antibody production has, from the beginning, been principally concerned with the response of the whole animal, and particularly with measurements of antibody concentration in the serum. The value of such studies is limited when we wish to use the immune response as a tool for investigating protein synthesis or the relation between structure and function of the cell.

The level of antibody in the serum of an animal at any particular moment is the resultant of complex processes of production, fixation to antigen deposits, excretion, and catabolism. Much attention has therefore been given in recent years to the experimental isolation of the antibody-forming process. Use has been made of more or less well-defined cell inocula injected into *x*-irradiated hosts (Roberts and Dixon, 1955; Harris and Harris, 1957; Chase and Wager, 1957; Stavitsky, 1958), of lymph nodes transplanted to hypogammaglobulinaemic recipients (Martin, Waite, and McCullough, 1957), and of incubation *in vitro* of tissue slices or fragments (Askonas and White, 1956; Thorbecke and Keuning, 1956; McKenna and Stevens, 1957; Stevens and McKenna, 1958; Askonas and Humphrey, 1958).

To supplement such studies it would be of value to be able to investigate quantitatively the features of antibody production by single cells, including the timing and rate of antibody formation, the turnover of intracellular antibody, and the cell content of antibody.

In this paper an approach is made to one aspect of the matter, a method for measuring the antibody content of individual cells. This work followed on an experiment reported earlier (Berenbaum, 1956). A full account of the technique is being prepared for publication elsewhere.

Materials and Methods

Immunization.—Rabbits were immunized by repeated injections of human serum albumin that had been precipitated with alum or mixed with an oily adjuvant. Injections were given by multiple routes and some were given subcutaneously into the ears, so that the regional lymph nodes were actively involved in the immune response.

Imprints.—At the fourth or seventh day after the last injection the rabbits were bled out under pentobarbitone. The parotid lymph node was cut and imprints of it made on glass slides. These were fixed in 95% ethyl alcohol at 37° C. for 15 minutes, as in the method of Coons, Leduc, and Connolly (1955) for demonstrating antibody by a fluorescein-labelling technique.

Labelled Antigen.—Radio-iodinated human serum albumin, obtained from the Radiochemical Centre, Amersham, was mixed with one-fifth of its volume of 10% (w/v) sodium iodide and 10 volumes of normal rabbit or guinea-pig serum. This was then mixed with an equal volume of saturated ammonium sulphate and centrifuged. The supernatant fluid was dialysed against saline at *p*H 7.0 and 4° C. until free of sulphate, made up with rabbit or guinea-pig serum to a final antigen concentration of 0.5 mg./ml. and centrifuged before use. These steps were taken to reduce non-specific surface adsorption of the labelled protein and remove radioactive contaminants.

At the time of use the antigen had a specific activity of 20 μC./mg.

Antibody-labelled Antigen Reaction.—The labelled antigen solutions were applied to the imprints in petri dishes lined by wet filter paper. After an hour the slides were washed in buffered saline for 10 minutes and placed in 80% ethyl alcohol. To block the antigen-antibody reaction specifically, 10 mg./ml. of unlabelled human serum albumin was added to the solution of homologous radio-iodinated antigen.

Autoradiography.—Lajtha's (1954) technique was used. Exposure was usually for two to four weeks at 4° C. After staining, the slides were mounted in D.P.X. and examined under an oil-immersion objective.

Results

Localization of Intracellular Antibody. — In imprints taken seven days after the last injection of antigen numerous developed silver grains were seen over plasma cells, but not over lymphocytes, granulocytes, mast cells, or reticulum cells, all of which could be easily identified in the stained imprints. Uptake of labelled antigen was blocked by excess of unlabelled human serum albumin. There was no uptake in imprints of animals im-

munized with heterologous protein antigens or bacterial vaccines or in unimmunized rabbits.

Estimation of Antibody Content per Cell.—After exposures of two half-lives (16 days) there were usually between 50 and 150 grains over and immediately around each cell that had fixed the labelled antigen. Occasional cells were seen with up to 300 grains. These figures are necessarily approximate; it is not possible to make accurate grain counts by visual means alone when there are 100 grains or more over individual cells.

Consider a cell that has produced 100 overlying grains in two half-lives. Since one I^{131} β-particle produces an average of 7.8 grains in stripping film (Cormack, 1955) and only half the emitted particles enter the film, the number of β-particles emitted from the cell in this time was $\frac{100}{1.8} \times 2$. In two half-lives three-quarters of the radioactive atoms will have released β-particles. Therefore the number of I^{131} atoms in the antigen fixed by the cell was $\frac{100}{1.8} \times 2 \times \frac{4}{3}$, or 148.

One Curie of carrier-free I^{131} weighs 7×10^{-6} g. (Francis, Mulligan, and Wormall, 1954). Therefore the weight of I^{131} in 1 mg. of radio-iodinated human serum albumin with a specific activity of 20 μC./mg. is $7 \times 10^{-6} \times 20 \times 10^{-6}$, or 140×10^{-12} g. The number of I^{131} atoms in 1 mg. of this antigen is therefore $140 \times 10^{-12} \times \frac{6.023 \times 10^{23}}{131}$.

Since 148 atoms of I^{131} were present in the cell, the amount of antigen fixed by the antibody it contained was $148 \times \frac{131}{140 \times 10^{-12} \times 6.023 \times 10^{23}} \times 10^{-3}$ g., or 2.1×10^{-13} g.

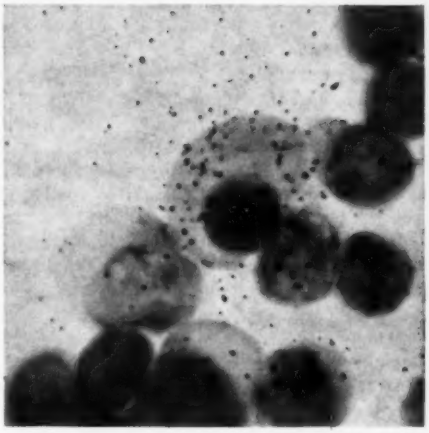
It remains to relate this quantity of antigen to the amount of antibody present. In solution the combining ratio of antigen and antibody depends on their valencies and concentrations, but the position is probably different when one of the reactants is insoluble. Coons *et al.* (1955) found empirically that fixing in 95% ethyl alcohol at 37° C. for 15 minutes made intracellular antibody insoluble without obviously impairing its combining power. In these circumstances the freely diffusible antigen molecules will tend to occupy all the reactive sites on the fixed antibody molecules unless the latter are so closely packed that there is appreciable steric hindrance. If sufficient antigen is present, the antigen-antibody combining ratio will thus depend only on the valency of the antibody. Taking the valency of rabbit antibody as 2 (Marrack, Hoch, and Johns, 1951; Plescia, Becker, and Williams, 1952), we may assume for our present purpose that one molecule of antibody (mol.wt. 165,000) has combined with two molecules of human serum albumin (mol.wt. 69,000). The amount of antibody in the cell under consideration is therefore $2.1 \times \frac{10^{-13} \times 165{,}000}{2 \times 69{,}000}$ g., or 2.5×10^{-13} g. Since between 50 and 150 grains were produced in two half-lives, the amount of antibody per cell ranged from 1.25 to 3.75×10^{-13} g., and in the occasional cell with about 300 grains there were 7.5×10^{-13} g. of antibody.

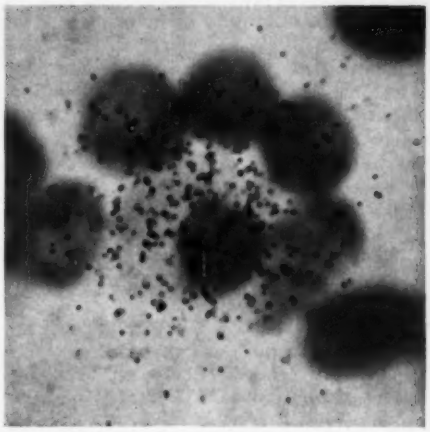
This is clearly only a first approximation. It is based on the assumptions that all or almost all the intracellular antibody is fixed by the alcohol treatment and that its combining power is not extensively impaired. These assumptions are at present being investigated. The errors they introduce are probably greater than those inherent in quantitative autoradiography. The latter are caused by the random nature of radioactive decay and by variations in the thickness of the autoradiographic film. However, if the assumptions as to the behaviour of alcohol-fixed antibody are not seriously at fault, the amount of intracellular antibody estimated is likely to be of the correct order.

Changes in Antibody Content during Cell Maturation.—When imprints were made four days after the last antigen injection, there was fixation of labelled antigen not only by mature plasma cells (Figs. 1, 2) but also by developing plasma cells and by cells so immature that they could not be identified as belonging to the plasma cell series (Figs. 3, 4). Immature cells had fixed only small amounts of antigen. There appeared to be an increase in antibody content with cell maturation, as maximum antigen fixation was seen only in mature plasma cells.

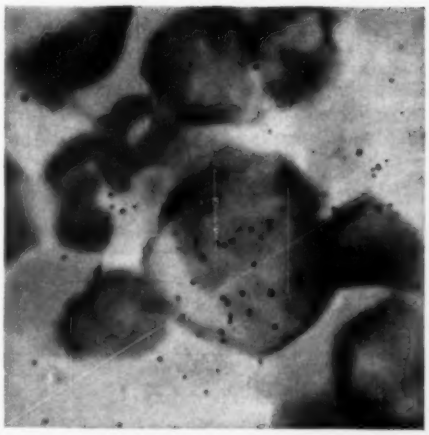
Discussion

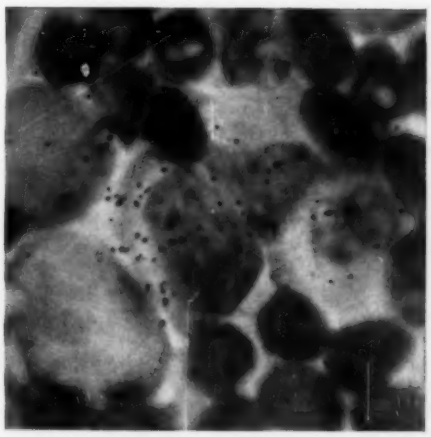
The specificity of this method for the demonstration of antibody is shown by the fact that positive results are obtained only by treating imprints from immune animals with labelled homologous antigen: they are not obtained when the animal is unimmunized or immunized with a heterologous antigen. Moreover, the reaction is





FIGS. 1 and 2.—Mature plasma cells.





FIGS. 3 and 4.—Immature cells.

Autoradiographs of antibody-containing cells in lymph-node imprints from a rabbit immunized with human serum albumin. Imprints made four days after the last antigen injection and treated with radio-iodinated human serum albumin. Exposure 28 days. Leishman–Giemsa. × 1,700.

blocked if excess of unlabelled human serum albumin is added to the radio-iodinated antigen, but not by excess of rabbit or guinea-pig serum proteins.

The finding that the antibody-containing cells are plasma cells accords with a considerable body of evidence that plasma cells contain and secrete antibody (Björneboe and Gormsen, 1943; Bing, Fagraeus, and Thorell, 1945; Fagraeus, 1948; Thorbecke and Keuning, 1953; Coons *et al.*, 1955; Askonas and White, 1956).

The primitive cells that appeared to contain small amounts of antibody may have been plasmablasts, or they may have belonged to the lymphoid follicles that, although not containing plasma cells, possibly contribute to antibody formation (Keuning and van der Slikke, 1950; Coons *et al.*, 1955; Leduc, Coons, and Connolly, 1955; White, Coons, and Connolly, 1955; Ortega and Mellors, 1957). The answer to this question depends on a more certain identification of cell types in imprints.

Results from three sources permit independent estimates to be made of the amount of antibody in a single cell.

(1) Roberts and Dixon (1955) transferred lymph node cells from immunized rabbits into the abdominal wall of *x*-irradiated recipients. From the rise in serum antibody in the recipients they calculated that each transferred cell had produced its own weight of antibody in about eight days. The weight of an average cell was 250×10^{-12} g., and therefore the rate of production was 30×10^{-12} g./24 hrs. Now the amount of antibody produced in 24 hours is equal to the cell antibody content multiplied by 24 and divided by the turnover time. Humphrey and Sulitzeanu (1958) found that the turnover time of rabbit antibody was two to three hours. On substituting these values in the above formula, the estimated cell antibody content works out at $25–37.5 \times 10^{-13}$ g. This is about 10 times higher than what is obtained by the autoradiographic method, but, considering the unknown factors operative in this, it may not be thought too great a divergence. However, as pointed out by Humphrey (personal communication), although the authors showed that there was no multiplication of cells at the site of injection, they did not exclude migration of donated cells to other sites and multiplication there. Their estimate of antibody production per cell may therefore be too high. It may be noted that Hašková and Mitchison (1958) found that spleen cells from immunized mice, which produce antibody after subcutaneous injection into non-immune hosts, left the injection site after a few days. However, antibody production continued, presumably by cells that had migrated to the regional lymph nodes.

(2) Humphrey and Sulitzeanu (1958) extracted intracellular antibody from the organs of hyper-immune rabbits and usually found about 0.05 mg./g. in lymph nodes. If we may assume that the number of antibody-producing cells in such lymph nodes was 1.0–10% of the total, then the concentration of antibody in the plasma cells was 0.5–5.0 mg./g., and the amount in a cell weighing 250×10^{-12} g. would therefore have been $1.25–12.5 \times 10^{-13}$ g.

(3) Nathans, Fahey, and Potter (1958) extracted the myeloma protein from a plasma-cell tumour of mice and found that it amounted to 1.7–5.4 mg./g. of tumour. If we assume that the tumour consisted entirely of plasma cells with an average weight of 250×10^{-12} g., each cell would have contained $4.05–13.5 \times 10^{-13}$ g. of myeloma protein.

Though results on the production of an abnormal protein by a neoplasm must be of limited relevance to work on antibody synthesis, the broad similarity between the two phenomena and the paucity of other quantitative results to some extent justify their use in this instance. The figures are summarized in the Table, from which it may be concluded that the autoradiographic method, although based on assumptions at present lacking adequate experimental support, is capable of giving a reasonable estimate of intracellular antibody content.

TABLE
ESTIMATES OF INTRACELLULAR ANTIBODY

Source of Data	Estimate
Roberts and Dixon (1955)	$25–37{\cdot}5 \times 10^{-13}$ g.
Humphrey and Sulitzeanu (1958)	$1{\cdot}25–12{\cdot}5 \times 10^{-13}$ g.
Nathans *et al.* (1958)	$4{\cdot}05–13{\cdot}5 \times 10^{-13}$ g.
Autoradiographic method	$1{\cdot}25–7{\cdot}5 \times 10^{-13}$ g.

Summary

Intracellular antibody may be demonstrated by treating imprints of lymph nodes from immune animals with a solution of radio-iodinated homologous antigen. Uncombined antigen is washed off and autoradiographs are made.

Antibody is present in plasma cells. Grain counts in the autoradiograph over individual cells permit the amount of combined antigen to be calculated. If certain assumptions are made, the amount of antibody per cell may be estimated.

According to these estimates, plasma cells appear to contain between 1.25 and 7.5×10^{-13} g. antibody per cell.

I am indebted to Drs. J. H. Humphrey and S. R. Pelc for helpful discussions. Skilled technical assistance was given by Mr. W. A. Cope. The photographs were taken by Mr. D. F. Boxall.

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THE CHAIRMAN'S SUMMING-UP

G. J. CUNNINGHAM

From the Royal College of Surgeons of England

This symposium has enabled us to view some aspects of the advances made in cellular physiology and pathology during the past 100 years. This progress has been particularly spectacular recently as a result of the development of new biochemical, biophysical, and microscopical techniques. In introducing this study we have been particularly fortunate in having a person like Sir Roy Cameron, whose researches and depth of knowledge have placed him at "the growing edge" of the subject.

Clinical pathologists will all have welcomed a study of the practical application of modern techniques as illustrated by exfoliative cytology. Familiarity with cytoplasmic and nuclear detail now enables malignancy to be diagnosed with accuracy in an increasingly higher percentage of cases. This is of great importance, for in some cases exfoliative cytology may be the only means of diagnosis for inaccessible growths. It is particularly gratifying that this method is being more extensively used in Great Britain by pathologists who, as a whole, were reluctant to employ a technique which presented many difficulties. As in all laboratory tests an adequate specimen is essential and this was often difficult to obtain as patients, though willing, were unable to co-operate. The discovery of certain aerosols which after inhalation result in the copious outpouring of bronchial secretion has done much to improve the quality of the specimen and so facilitate diagnosis.

Although at one time bacteria tended to be regarded as distinct from other cells, recent work on their nucleic acid content has done much to indicate that their nature is essentially the same. Their biochemical content has become increasingly more important in the investigation of metabolic blockage by antibiotic substances. While new antibiotics keep appearing, agents active against the smaller viruses are more elusive and the discovery of "interferon" by Isaacs and his colleagues is of the first importance. This substance, produced when certain viruses are grown in tissue culture, causes metabolic blockage of certain viruses, and it remains to be seen whether any therapeutic application will develop from this observation *in vitro*. This is only one of the applications of tissue culture, a technique for which we owe so much to Dr. Honor Fell. Apart from the value of observing living or growing cells, embryonic organ culture has also been developed, whereby the influences of chemical factors on growth can be assessed. It is likely that by this means light will be thrown on the mechanism of such processes as regeneration and repair. In recent years morbid anatomy and bacteriology have seemed to stand less apart following the demonstration of tissue immunity in attempts at grafting tissues to aid the repair process. Although many differences exist between antitoxic and tissue immunity the presence of transplantable cell antigens has been demonstrated in tissue homografts. Those who have worked on this subject are well aware of the complexity of such antigens, and the recent discovery of the agar-diffusion plate method by Ouchterlony may do much to aid future workers in resolving antigenic complexes. The recent dramatically successful kidney transplant in America in one of identical twins suffering from uraemia and chronic nephritis does much to indicate the wonderful future which lies ahead if only homovital homografts can be made a practicable proposition.

The biochemical advances need no emphasis, for they are familiar to all. Mitochondria are now known to be sensitive indicators of subtle changes in intracellular environment, and may later take on forms described many years ago as degenerative changes. Future work may do much to indicate the nature of metabolic requirements in various kinds of specialized cells. Much information in the difficult field of protein chemistry has appeared and the briefest reference to it is bound to include tribute to the outstanding work of Sir Frederick Gowland Hopkins. He indeed would be most gratified at the recent outstanding discoveries by *x*-ray crystallography of the molecular structure of insulin by Sanger and of myoglobin by Kendrew. A special class of proteins, namely enzymes, have appeared in increasing numbers, and the recent histochemical techniques give promise of future discoveries of the first magnitude and importance. The concept of localizing enzymes within individual mitochondria is indeed a great step forward. It is quite possible that combined techniques employing histochemistry

and *x*-ray microscopy may do much towards the precise location of chemical substances within the cell, thus enabling the identification of the sites of metabolic action. In the midst of these exciting concepts, it is reassuring to learn of ways in which the recognition of enzymes can be put to use in clinical practice. The finding of abnormal enzyme contents of the blood in diseases causing cellular damage and necrosis gives promise for the future of similar valuable diagnostic aids.

As knowledge accumulates at increasing speed it becomes especially important to pause from time to time and assess the worth of all the evidence. The present year, when interest in so many places seems to centre on the living cell, seems particularly appropriate for this purpose. The World Fair at Brussels devoted a section to the living cell, and at the American Medical Association meeting at San Francisco attention was attracted by means of a large plastic model approximately one million times the size of the average cell. In these times many of us feel alarmed at the ever-widening horizons of pathology and the numerous branches of the subject which threaten to become severed from the parent stem. It is therefore reassuring to see, as we have seen to-day, pathologists, cytologists, bacteriologists, and chemical pathologists joining forces and finding common ground in the study of the cell. When we speak of the cell, which is so small, we should at the same time realize that it is large enough to encompass all groups of workers in pathology. Indeed, when we speak of the cell, we are speaking of pathology as a whole. When yet another step forward towards the full understanding of the cell is made it will only be as the result of our united efforts integrated towards a common goal.

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ASSOCIATION OF CLINICAL PATHOLOGISTS: 61st GENERAL MEETING

The 61st general meeting was held at the Royal College of Surgeons, London, on October 2, 3, and 4, 1958.

The papers delivered in honour of Virchow's work on cellular pathology are published in full in this issue, and summaries of the other papers follow.

Congenital Pulmonary Lymphangiectasis

K. M. LAURENCE (Hospital for Sick Children, London) said that congenital pulmonary lymphangiectasis was characterized by a progressive, unrelieved cyanosis which generally leads to death within 24 hours of birth. At necropsy the bulky and inelastic lungs, with an exaggerated lobular pattern, prominent subpleural lymphatic vessels and cysts, are diffusely honeycombed with fluid-filled cysts measuring up to 4 mm. in diameter. Histologically these endothelium-lined cysts are situated in abundant young connective tissue under the pleura and the interlobular septi show a close relationship to blood vessels and bronchi. The lung parenchyma itself is mature. The position and structure of these cysts, which are shown by reconstructed serial sections to be part of an intricate network of irregular inter-communicating channels, indicate that they are lymphatic in origin.

A developmental disorder is suggested by its frequent association with other severe anomalies and by the early onset of symptoms. It is probably caused by a continued growth of the lung with the tissue elements maintaining their 16-week inter-uterine proportions, when the lymphatics are very much more prominent than at term. The progressive cyanosis might be due to cysts taking up some of the available thoracic space or pressing upon vessels, but more likely to the inelasticity of the lung leading to circulatory embarrassment.

The anomaly is certainly more common than the few previous reports suggest. An incidence of 1 in 100 to 150 perinatal necropsies is indicated by the 13 cases discussed. (This paper will be published in full in the *Journal*.)

Pathology of Familial Pulmonary Hypertension

J. TREGILLUS (Darlington) and P. N. COLEMAN (Northallerton) gave details of the morbid histology in three fatal cases of primary pulmonary hypertension in a brother and two sisters (Coleman, Edmunds, and Tregillus, 1959). There was extensive intimal proliferation in the pulmonary arteries which had caused serious occlusion in the smallest branches. It was probable that all the intimal proliferation was the result of previous thrombosis.

The hypertension was almost certainly genetic in origin, but the mode of operation of the genetic defect remained in doubt. No congenital anomaly could be found and hypertension was explained as the result of an inherited functional defect causing hypertonia in the muscular arteries. There was histological support for this in the findings of contracted arteries in the sections. Symptomless functional hypertension may have been present for many years, finally bringing about changes in the pulmonary arteries favourable to the development of thrombosis and intimal fibrosis. The obstruction then became too much for the functional reserve of the right heart and symptoms of failure appeared for the first time. In the two sisters, pregnancy may have proved an additional stress leading to an earlier development of thrombosis.

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Infantile Cardiomegaly: the Infantile Type of Endocardial Fibroelastosis

R. I. K. ELLIOTT (Royal Sussex County Hospital, Brighton) said that the infantile form of endocardial fibroelastosis was distinguished by generalized hypertrophy of the heart with dilatation of the left ventricle and marked thickening of the mural endocardium. Affected children are, typically, healthy and normal until their terminal illness; this may take the form of progressive congestive failure, but is more commonly abrupt, catastrophic collapse and death suddenly occurring in the course of a mild febrile upset.

It is unlikely that the endocardial thickening is anything more than an incidental finding; the defect, as Brigden (1957) has suggested, is probably in the myocardium. Histological findings tend to support this; there is interfascicular and interfibrillary oedema and longitudinal banding of the sarcoplasm of the myofibrils. The condition appears to be familial, a history of unexplained sudden death in infancy among the siblings of either the child or its parents being obtained in three of the six cases in this series.

The present terminology, with its emphasis on fibroelastosis, is misleading; the old description "congenital idiopathic hypertrophy" was better; but as an alternative, more in line with modern views, the name "infantile cardiomegaly" is suggested.

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Studies on Endogenous Lipid Pneumonia and Blood Lipid Levels in Cortisone-treated Rabbits

T. J. MORAN (Presbyterian Hospital, Pittsburgh, U.S.A.) described studies of the effect of cortisone on the development of bronchiolitis obliterans in rabbits given intratracheal injections of 1.0% nitric acid which showed that cortisone prevented or modified the experimental lesions of bronchiolitis obliterans but did not influence the death rate from acute pulmonary oedema or pneumonia compared with control animals. During the course of the study a peculiar form of "endogenous lipid pneumonia" was observed in cortisone-treated animals which had been given intratracheal nitric acid. The lipid material in this form of "endogenous lipid pneumonia" varied greatly in size and was chiefly extracellular as compared with the endogenous lipid pneumonia observed in animals not treated with cortisone and in human examples of this condition. The relation of this pneumonia to various causes was discussed: (1) Overloading due to high blood lipid levels caused by cortisone; (2) possible interference with macrophage ingestion of the lipid; (3) interference with the macrophage motility and activity; (4) possible physical or chemical change in the lipid itself.

In the discussion it was pointed out that rabbits were very susceptible to cortisone, and that the picture might bear no relationship to the human disease.

There was also some discussion on "silo fillers' disease," where the workers are exposed to NO_2, which occurs in high concentration within two days of the silo being formed, and the differentiation of this from farmer's lung, which is probably due to fungus infection, and possibly associated with "silo emptiers' disease."

Nephritis Associated with Streptococcus Pyogenes Type 12

D. J. H. PAYNE and D. A. SLADE (Northallerton) gave an account of an epidemic of nephritis in north Yorkshire. Five cases occurred in a small village in 1957, during the first two months of the year. Throat and nasal swabs from the local schools showed that there was a high incidence of *Streptococcus pyogenes* type 12 infection in these schools. Urines of the children showed albumin, red cells, and casts in some cases, which, when compared with urines from children in schools 25 miles way, indicated kidney damage. In all, 43 children, 10 of whom had type 12 strains in their throats, had evidence of kidney damage. Sixteen months later, seven out of 43 still have abnormal findings—five with albumin, one with red cells and casts, and one with red cells only. No more cases occurred until December, 1957. From then until the present time (September, 1958), cases have occurred in the Northallerton area and York mainly during the January–March period as before. Twenty-four cases from the Northallerton area yielded 10 type 12 strains and, in York, nine type 12 strains were isolated from 25 cases and type 22 from another. In all more than 60 cases of acute nephritis have occurred in the area. The ages ranged between 4½ and 50 years, 25% being over 21 years of age. The male to female ratio was 2 to 1. Only four out of 32 did not have a history of sore throat or otitis media. Mildness of the initial symptoms was a feature, as was a shorter latent period between infection and the onset of nephritis, than has been described. Follow-up of the cases is proceeding and might, in time, throw light on the aetiology of chronic nephritis.

In the discussion which followed it was suggested that serological follow-up of, for example, ASO titre might also give some indication of the imminence of complications to this infection.

The Control of Cross-infection by Formaldehyde Disinfection of Blankets

H. CAPLAN (Highlands General Hospital) said that regular sterilization of blankets and bed-side curtains was an essential measure in the prevention of cross-infection. Formaldehyde possesses a direct bactericidal activity which is enhanced by moderate heat and by a vacuum (which increases the penetration of the gas).

Using a commercially available formaldehyde generator (supplied by Messrs. Manlove, Alliott and Co., Ltd., London, S.W.1) attached to the ordinary hospital autoclave, blankets are sterilized by exposure to formaldehyde vapour in a vacuum of 15 in. Hg at 55° to 60° C. for 20 minutes. Hot air is then blown through to remove the smell. Up to 50 blankets can be sterilized at a time in an autoclave chamber of 120 cu. ft. capacity. Following the introduction of this technique cross-infection in a male general surgical ward of 25 beds was eliminated. There was one wound infection in the ensuing six months compared with 17 in the preceding six months. The incidence and severity of urinary infections following prostatectomy were markedly diminished from 70 to 100% down to as low as 20% and the average stay in hospital after prostatectomy was reduced from 60 to 20 days. The consequent saving on drugs alone meant that the apparatus paid for itself in less than six months. Blankets have now been sterilized fortnightly for over a year and have not shrunk or become matted and formalin dermatitis has not been experienced.

The Antibody Content of Single Cells

M. C. BERENBAUM (Greenford) described an autoradiographic technique for localizing and measuring antibody in individual cells. The paper is published in full on page 543.

Observations in Bone Marrow Transplants in Man

J. G. HUMBLE, K. NEWTON, and N. H. KEMP (Westminster Hospital, London) discussed bone marrow transfusions, and said that the two features which make it difficult to apply well-known tech-

niques used with animals to human transfusions are lack of precise knowledge of the effects of ionizing whole body irradiation in man and the difficulty of obtaining sufficient cells for transplanting. For this reason they tried the effects of local irradiation, for example, of the ilium, and using the transplant as an infusion of cell suspension into the aorta. In two cases there was certainly improvement. In one case of leukaemia there appeared to be a definite slowing down of the process, and in another case the irradiated zone on examination had become cellular. The appearance of female clubs in the polymorphs after transfusion of female blood into aplastic marrow suggested in one case that the transplant had "taken." It was noted that the procedures were well tolerated by the patients, most of whom were extremely ill.

JOINT MEETING WITH THE ASSOCIATION OF CLINICAL BIOCHEMISTS

This meeting was a symposium on paper chromatography, at which Professor C. E. Dent took the chair.

Chromatography of Androgens, Oestrogens, and Progestogens

R. V. SHORT (A.R.C. Unit of Reproductive Physiology and Biochemistry, Cambridge) said that the problem of steroid determinations in the urine of domestic animals was complicated by qualitative differences in the metabolites excreted by some species and the presence of interfering substances in the urine extracts. In addition, the major excretory pathway for some steroid metabolites may be via the gastro-intestinal tract. These difficulties have emphasized the need for a simple technique that is capable of measuring the active steroids in the peripheral blood of any animal.

Such a method has already been described for the determination of progesterone in peripheral blood (Short, 1958a). With a few minor modifications, this method can be adapted for the simultaneous determination of androstenedione, testosterone, oestrone, oestradiol-17β, oestriol, and probably corticosterone and hydrocortisone (Short, 1958b). The basic steps of the modified procedure are as follows:

(1) Titration of the plasma to *p*H 10.0 with *N* NaOH.

(2) Extraction with ether.

(3) Ether extract evaporated to dryness, partitioned between light petroleum and methanol.

(4) Application of methanolic extract to a paper chromatogram.

Paper chromatography is carried out in the Bush system appropriate for the steroids to be estimated. The steroids are eluted from the paper and measured in the spectrophotometer. The Kober reaction is used for the oestrogens, and the ultra-violet absorption at 240 mμ for the $\alpha\beta$-unsaturated androgens and progestogens. Using a microcell attachment, this limits the sensitivity to 0.5 to 1 μg. steroid. With the application of fluorimetric techniques, it should be possible to achieve a ten- to hundred-fold increase in sensitivity. This should permit the determination of these steroids in a small sample of peripheral blood.

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—— (1958b). *Mem. Soc. Endocr.* In the press.

The Assessment of Disordered Indole Metabolism Using Paper Chromatography

M. SANDLER (Queen Charlotte's Maternity Hospital, London) said that assay of biological fluids for indoles by paper chromatographic techniques could only be, at best, roughly quantitative. The reasons given for this view included the lability of some of these compounds during electrolytic desalting procedures, their susceptibility to atmospheric oxidation, certain specific drawbacks of the solvent systems commonly used, and the variability of colours obtained with developing reagents.

Qualitative studies, using two-dimensional techniques especially, are of great value in clinical practice, however. Diagnostic patterns may be obtained in the carcinoid syndrome, Hartnup disease, phenylketonuria, melanogenuria, and other disorders.

The Fractionation of Serum Proteins by Ion Exchange Chromatography

M. P. TOMBS, K. G. COOKE, and N. F. MACLAGAN (Westminster Hospital, London) described a method which had been developed for the fractionation of serum proteins into five groups (P, A, B, G, and L in order of elution) on carboxy-methyl cellulose columns (Peterson and Sober, 1956). Protein was estimated in the eluent by the ultra-violet absorption at 280 mμ. Carboxy-methyl cellulose was chosen because it was possible to obtain higher flow rates of eluting buffer than with diethyl amino ethyl cellulose, so that a serum required about three hours for examination. Group P contained mainly α_1 globulins. Albumin was fractionated between groups A and B, but it was shown by re-chromatography that the two forms of albumin were completely interconvertible. They may bear some relationship to the electrophoretically distinguishable isomeric forms of albumin reported recently. The α_2 globulins were distributed throughout the elution system, and β globulins occurred mainly in group B and γ globulins in B and G. The areas of the peaks bore a strict quantitative relationship to the amount of protein eluted, and can be used for comparative purposes, provided due allowance is made for the different specific absorption of the various components at 280 mμ. It seems that resolution into electrophoretically homogenous components is very unlikely using a single-run technique, but that re-chromatography of groups obtained in a rough elution system such as this one leads to very high resolution. There is no upper limit to the amount of serum which can be employed.

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Uro-pepsinogen Excretion in Patients with Gastric Lesions

J. SCHRAGER (Wigan) discussed uro-pepsinogen excretion in patients with gastric lesions. The uro-pepsinogen excretion was estimated in 232 healthy adults, 188 patients with active duodenal ulcer, 66 patients with gastric ulcer, and 90 patients with gastric carcinoma. At least three 24-hour specimens of urine were examined in each individual. The available data show that the range of uro-pepsinogen excretion in healthy adults falls between 36 and 90 μg. per hour with an average mean output of 70 μg. per hour (using Armour pepsin as standard). It remained constant within 10% for each person. In many cases the tests were repeated three or four times at monthly intervals, without significant change, in spite of wide variation of urine volume. Patients with duodenal ulcer had uro-pepsinogen excretion well above normal with a mean output of 150 μg. per hour. The excretion in patients with gastric ulcer was lower than that of duodenal ulcer patients, but significantly higher than the controls, with a great variation in output, 35–270 μg. per hour. It is suggested that this is due to the pathological condition of the gastric mucosa. Histological examination of the stomachs of patients who were operated on and whose uro-pepsinogen level was previously estimated revealed widespread gastritis and metaplasia. It was possible to correlate the uro-pepsinogen excretion with the state of the gastric mucosa. The uro-pepsinogen excretion in patients with gastric carcinoma was well below normal, and a considerable number excreted no uro-pepsinogen at all. The mean uro-pepsinogen excretion in this group was 26 compared with the normal 70 μg., and 126 μg. in patients with duodenal ulcer. It is suggested that uro-pepsinogen excretion may prove helpful in differentiating between benign and malignant lesions, and may help in the discovery of early malignant changes. The disappearance of uro-pepsinogen from the urine on repeated examination suggests widespread changes in the mucosa and warrants further examination.

An Immunochemical Analysis of the Proteins of Normal Urine not Present in Blood

G. H. GRANT (Royal Salop Infirmary, Shrewsbury) described the immunochemical analysis of normal urine proteins by the agar gel diffusion and immuno-electrophoretic methods. Rabbit antisera prepared against normal urine colloids, against normal semen, and against the salt-precipitated mucoprotein of Tamm and Horsfall respectively were used after absorption with normal serum.

About 11 antigenic components not present in normal blood serum were detected in normal urine. Some appear to be specific to the urinary tract; others are common to male urine, female urine, and semen, and so might be excreted by the urethral glands. In addition traces of prostatic proteins occur in the male.

The mucoprotein of Tamm and Horsfall appears to be the principal urine protein not detected in blood. It arises from the upper urinary tract and is a mixture of immunochemically similar proteins of varying electrophoretic mobility.

Published methods for the quantitative analysis of urinary mucoproteins measure the carbohydrate content of protein mixtures rather than that of a single fraction.

Comparison of Protein-bound Iodine Determinations with Radioactive Test in Thyroid Disease

K. STEINITZ (Rothschild Hospital, Haifa, Israel) discussed a modification of Barker's incineration method for routine use. Incubation is at 24±0.1° C. for 30 minutes in an ordinary well-insulated water-bath. Colorimetry is done without the internal standard described by Barker. The two-hour and the 24-hour uptake, the eight- to 24-hour urinary excretion and the PBI^{131} after 48 hours are compared separately with the PBI^{127} values. The two-hour uptake shows a good correlation in clear cases of hyperfunction, a bad correlation in normal, and a worse one in cases with non-toxic goitre or other states of iodine avidity. About three-quarters of this group cannot be diagnosed correctly. The 24-hour uptake, to-day considered as the least valuable of all I^{131} tests, gave in 39 of 40 cases of non-toxic goitre or iodine avidity an abnormal increase, together with normal PBI^{127} values. The eight to 24-hour urinary excretion of I^{131} gives results comparable with the two-hour uptake, i.e., relatively good results for clear hyperfunction, and a false classification of about 70% of euthyroid cases of iodine avidity. The 48-hour PBI^{131}, though known to be influenced by the rate of turnover of the organic iodine in the thyroid, proved to be superior to the previous three methods. Its correlation with PBI^{127} is much better, and even in the bad group with iodine avidity the error is less than 20%.

The Estimation of Oxalic Acid in Urine and Other Biological Material

A. HODGINSON and P. M. ZAREMBSKI (The General Infirmary at Leeds) reviewed the existing methods for the estimation of oxalic acid in urine and described a new procedure based on that of Powers and Levatin (1944).

The principle of the method is as follows: Urine is acidified with hydrochloric acid and half-saturated with ammonium sulphate. The gelatinous precipitate formed after standing is removed by filtration and the filtrate is extracted continuously with peroxide-free ether. The extracted oxalic acid is precipitated as the calcium salt, and reduced to glycollic acid by boiling with zinc powder and sulphuric acid. Glycollic acid is estimated colorimetrically by heating with chromotropic acid.

Recovery of oxalic acid from aqueous solution and from urine was found to be 98±2%. The procedure can be applied, with suitable modification, to the

determination of oxalic acid in foodstuffs and renal stones.

The daily excretion of oxalic acid by 39 normal adults on a balanced diet containing 60 to 80 mg. of oxalic acid daily ranged from 9.0 to 23.8 mg. per 24 hours. The urinary excretion of oxalic acid was found to vary directly with the intake of oxalic acid and inversely with the intake of calcium. Excretion also varied directly with the purine content of the diet, and a close correlation was found between the urinary excretion of oxalic acid and uric acid.

The authors wish to thank Dr. J. Dawson and Mrs. K. Rodgers, Department of Biochemistry, University of Leeds, for their advice in the initial stages of this investigation.

REFERENCE

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The Inheritance of an Idiopathic Low Plasma Pseudocholinesterase Level

H. LEHMANN, V. PATSTON, and E. RYAN (St. Bartholomew's Hospital, London) discussed the inheritance of an idiopathic low plasma pseudocholinesterase level. The short-term action of the muscle relaxant suxamethonium is due to its rapid destruction by pseudocholinesterase. If the plasma level of this enzyme is low, the response to the drug is prolonged. The usual causes of this are liver disease and to a lesser extent general debility. Very occasionally patients were observed with an abnormally long response to suxamethonium where the low enzyme level was without obvious aetiology. Investigation of the families of such people led to the discovery of healthy persons with abnormally low pseudocholinesterase levels. It was suggested that the likelihood of finding these people by chance would be well below 1 in 1,000. In an attempt to get information on the mode of inheritance it was assumed that a very low pseudocholinesterase level up to 35 units (Warburg) represented the homozygous condition for an abnormal gene, and that the high normal level above 90 units indicated the normal homozygote. It remained to be seen whether within the intermediate range 36–90 units the heterozygote could be differentiated from the two homozygotes. Kalow and Staron have described an atypical pseudocholinesterase and have examined the mode of its inheritance, but it is not yet certain whether the homozygotes for the gene determining the abnormal enzyme correspond with the abnormal homozygotes of the present study. Earlier findings seemed to be consistent with the assumption that the inheritance of a recessive gene might be responsible for an idiopathic enzyme level less than 36 units. Allott and Thompson, however, described a family where the enzyme levels in three children and one sister of a male propositus were roughly halfway between those seen in the propositus and in his wife, who had normal levels. They suggested that a single gene might find expression in the phenotype. The normal plasma pseudocholinesterase level has a range of 61 to 120 units. A level within the 36 to 60 range in otherwise normal persons should therefore indicate the heterozygous state. Random findings of such people is unusual, but amongst 72 relatives of 14 propositi 16 of them were discovered (22%). The 72 relatives were composed of the propositi's children and grandchildren, their siblings with their children and grandchildren, and their parents with their siblings. Forty-three of the remaining 56 relatives showed values within the normal 61–120 range. This range might well be subdivided into one greater than 90 units definitely indicating the normal homozygous state, and another 61–90 units within which both normal homozygotes and heterozygotes could be found. The following states could then be presumed:

Less than 36 units = abnormal homozygotes
36–60 units = heterozygotes
61–90 units = heterozygotes and normal homozygotes
Greater than 90 units = normal homozygotes

If this assumption is correct no parents of children of persons with an enzyme level less than 36 units should show a level greater than 90 units, but some should be found amongst their siblings, the number depending on whether their parents were all heterozygotes or included some abnormal homozygotes. By studying the families of 14 propositi data were obtained which appeared to fulfil these conditions and which might allow the conclusion that the inheritance of the plasma pseudocholinesterase level is determined by two allelic genes, neither of which shows dominance.

People with Plasma Pseudocholinesterase Less than 36 Units	Relatives		Plasma Pseudocholinesterase (Warburg Units)			
	Group	No.	Less than 36	36–90	61–90	More than 90
17	Parents and children	34	1	12	21	0
13	Siblings	26	11	3	9	3
8	Spouses	8	0	0	4	4

A Blood Serum Test for Leukaemia and Neoplasia

D. STARK MURRAY and J. M. DALY (Group Laboratory, Kingston-on-Thames) reported a theory of the mechanism involved in leukaemia which postulates a flaw in D.N.A. production in the bone marrow through a histidine or imidazole compound. This would have the double action of removing zinc ions which normally inhibit D.N.A.se and of acting directly as an accelerator of D.N.A.se activity. A search for this substance revealed a compound, probably a conjugate since it can be detected only after hydrolysis in a hot alkaline solution (0.5 ml. serum boiled for 30 seconds in 1 ml. 20% Na_2CO_3), and which appears to be present in excess in neoplastic cases. It is measured by adding 2.5 ml. of diazo

reagent and reading with an Ilford filter 404 in the colorimeter against a blank prepared at the same time. No false negatives have been obtained and only two proven false positives out of 346 tests. The test appears very reliable as a diagnostic aid. The normal range is very narrow and distinct from abnormal. Highest "neoplastic" readings are found in myelogenous leukaemia; in lymphatic leukaemia these are not so high. In carcinoma the figure is always above normal but very variable although all carcinomas give a positive reading. It is possible that some other forms of malignancy do not give positive results, but sufficient cases have not yet been tested. The test is upset by raised urea figures, and false positives are probably related to liver damage. The test may prove to have a value in assessing the effect of different forms of treatment of the neoplastic diseases.

Association of Clinical Pathologists Broadsheets

The following broadsheets (new series) are published by the Association of Clinical Pathologists. They may be obtained from **Mr. G. S. Corden, Swan Press Ltd., 20 Bakers Row, Farringdon Road, London, E.C.1,** price 1s. each.

No.	Title	Date	Author
1	The Disc Technique for the Rapid Determination of Bacterial Sensitivity to Antibiotics	1952	R. W. Fairbrother
2	Determination of Sensitivity of *M. tuberculosis* to Streptomycin	1952	R. L. Vollum
3	The Detection of Barbiturates in Blood, Cerebrospinal Fluid, Urine, and Stomach Contents	1953	L. C. Nickolls
4	The Estimation of Carbon Monoxide in Blood	1953	D. A. Stanley
5	The Identification of Reducing Substances in Urine by Partition Chromatography on Paper	1953	G. B. Manning
6	The Paul-Bunnell Test	1954	R. H. A. Swain
7	The Papanicolaou Technique for the Detection of Malignant Cells in Sputum	1955	F. Hampson
8	Investigation of Haemorrhagic States with Special Reference to Defects of Coagulation of the Blood	1955	E. K. Blackburn
9	Daily Fat Balance	1956	A. C. Frazer
10	Mycological Techniques: (1) Collection of Specimens	1956	R. W. Riddell
11	Mycological Techniques: (2) Cultural Isolation	1956	R. W. Riddell
12	Techniques for Demonstrating L.E. Cells	1956	J. V. Dacie and L. S. Sacker
13	The Identification of Serotypes of *Escherichia coli* Associated with Infantile Gastro-enteritis	1956	Joan Taylor
14	The Determination of Serum Iron and Serum Unsaturated Iron-binding Capacity	1956	Arthur Jordan
15	The Estimation of Faecal "Urobilinogen"	1957	C. H. Gray
16	Preservation of Pathological Museum Specimens	1957	L. W. Proger
17	Cultural Diagnosis of Whooping cough	1957	B. W. Lacey
18	The Rose-Waaler Test	1957	C. L. Greenbury

BOOK REVIEWS

Muir's Textbook of Pathology, 7th ed., revised by D. F. Cappell. (Pp. xx+1,201; 733 figures. 70s.) London: Edward Arnold. 1958.

So excellent and widely known a textbook as Muir's needs little write-up from any reviewer, and, in the capable hands of Professor Cappell, the traditions of the work as a contemporary survey of the subject are admirably maintained. Since the last edition was published in 1951 a good deal of new material has collected. This has called for radical revision of the text, and the modest addition of 111 pages has meant the curtailment of topics of decreasing clinical importance, but a good proportion of these extra pages is to be accounted for by the addition of 100 illustrations.

The transfer of the section on repair of fractures to the more general chapter on inflammatory processes, the addition of a note on the collagen diseases, and the modernization of the section on fluid and electrolyte balance are but random examples testifying to the thoroughness of the revision. The treatment of diseases of the blood, which includes the more recent immunological researches in haematology, is particularly to be commended. In general this is a text upon which the undergraduate student may confidently rely.

DOROTHY S. RUSSELL.

An Introduction to Blood Group Serology. By Kathleen E. Boorman and Barbara E. Dodd. (Pp. viii+317; 30 figures, 43 tables. 40s.) London: J. and A. Churchill. 1957.

It is always difficult, when undertaking a new book, to decide on the exact scope and then to keep the book within the set limits. Thus many books on blood serology become so involved with theoretical concepts that the practical side is lost sight of, or else the authors are so determined to make the book simple to follow technically that it becomes inadequate. The authors of this new book on blood serology have found a happy balance. In spite of the title they have kept their theoretical aspects to basic accepted principles and have devoted themselves to a carefully arranged plan of introduction to blood group serology which covers nearly all the known and accepted techniques, succinctly described, so that anyone can follow when reading for the first time of a new technique and can as easily use them as instructions for the bench. There is a most helpful, critical, comparative comment with each method. The authors come down firmly in favour of certain techniques, and the reasons are clearly stated. They have introduced a method of identifying the techniques by numbers, so that when describing more advanced methods the simpler techniques are identified by the number, thus avoiding repetition. Altogether 100 techniques are numbered and described, and the book ends with an appendix of 13 plain procedures such as identification of antibodies, paternity tests, investigation of a suspected haemolytic transfusion reaction, etc., in which the technique for each group is indicated by the number, thus compressing into a few pages all the more usual laboratory techniques required. In all an admirable book for the routine laboratory worker, and no laboratory where blood grouping is carried out will in the future be without its "Boorman and Dodd," whose names are now as euphonically linked in blood serology as "Whitby and Britton" in haematology.

A. G. SIGNY.

The Cerebrospinal Fluid: Production, Circulation, and Absorption. A Ciba Foundation Symposium edited by G. E. W. Wolstenholme and Cecilia M. O'Connor. (Pp. xii+335; 141 figures. 50s.) London: J. and A. Churchill. 1958.

This volume contains the 17 contributions presented at the Ciba Foundation symposium on the "Production, Circulation, and Absorption of the Cerebrospinal Fluid" which was organized by Dr. H. N. H. Genese and held in May, 1957. They are in the nature of review articles and contain a résumé of the most recent research of their authors. In all there were, under the chairmanship of Professor G. A. G. Mitchell, 25 participants, and they ensured a series of lively discussions following each of the papers. Finally there was a general discussion. The editors are to be congratulated once more in so realistically representing these instructive debates to the reader: they have evolved a technique which is eminently of service to a wide range of interested persons.

There are so many matters of absorbing interest in these papers that it is impossible to comment on any of them individually other than to say that they well repay study by those who are concerned with the physiological mechanisms of the central nervous system and in particular by those who are interested in the clinical pathology of the cerebrospinal fluid.

W. H. MCMENEMEY.

BOOKS RECEIVED

(Review in a later issue is not precluded by notice here of books received.)

Studies in Fertility 1956. Vol. VIII of the Proceedings of the Society for the Study of Fertility. Papers read at the London Conference, 1956. Edited by R. G. Harrison. (Pp. x+138; illustrated. 25s.) Oxford: Blackwell Scientific Publications. 1957.

Maladies-Vedettes (Maladies d'Avenir, Maladies Quotidiennes, Maladies d'Exception). By Fred Siguier. (Pp. 476; 36 figures; Fr. fr. 3,500.) Paris: Masson. 1957.

The Diagnosis and Treatment of Infections. By D. Geraint James. (Pp. viii+234. 30s.) Oxford: Blackwell Scientific Publications. 1957.

Rheumatoid Arthritis. By Charles L. Short, Walter Bauer, and William E. Reynolds. (Pp. x+480; illustrated. 55s.) Harvard University Press (London: Oxford University Press). 1957.

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